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AGRICULTURAL RESEARCH INSTITUTE
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CLXXVI. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

XVII. THE ACTION OF SOME DERIVATIVES OF PHENAZINE, QUINOLINE AND PYRIDINE ON THE PASTEUR REACTION.

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In the course of work on the action of reversible oxidation-reduction dyes upon tissue metabolism it was found [Dickens, 1935, 1, 2] that the negative dyestuff phenosafranine (E_h at pH $7 = -0{\cdot}252$ v.) even in 10^{-5} M solution could inhibit completely the Pasteur mechanism in normal and tumour tissues. This action has been further studied in the present paper and has been found to extend to other substances showing a certain chemical relationship with phenosafranine.

Methods.

The experiments were made at 37·5° in the Warburg apparatus, using tissue slices suspended in bicarbonate- or phosphate-Ringer solution at pH 7·4 containing 0·2 % glucose. The duration of the experiments was usually 1 hour, unless otherwise stated. In these experiments it was desirable to follow the time course of the metabolism, and for this Warburg's 2-vessel method is most suitable. For measurement of R.Q. the method of Dickens & Šimer was used.

Phenosafranine (B.D.H.) could be dissolved directly in the solution, but many of the substances added are sparingly soluble in the cold and were therefore dissolved with warming and grinding in the salt solution before adding bicarbonate or glucose; even in the high dilutions used, solution of some of the reagents was incomplete.

Phenosafranine.

Added to slices of normal highly glycolysing tissues (brain cortex, extraembryonic membranes) or tumour tissue (JRS, Walker 256 carcinoma of rat), phenosafranine in appropriate concentration has little effect on respiration, but causes an immediate increase of aerobic glycolysis (Table I). The optimum concentration is close to 10^{-5} M for brain and a little higher for the other tissues. Above the optimum concentration the respiration is rapidly poisoned, whilst at lower concentrations (10^{-6} M) there is no change from the normal metabolism. The R.Q. of brain is not appreciably depressed below the carbohydrate level by 10^{-5} M phenosafranine, and chemical estimation by Clausen's method showed that the acid formed is lactic acid (Exp. 3, Table I). In some experiments a slight increase of respiration occurred with phenosafranine; however, the respiration of brain fell rather rapidly with concentrations of 5×10^{-5} M or a little above, but not to the same extent with the other tissues in Table I. The aerobic glycolysis in presence of phenosafranine rises until it becomes nearly equal to the anaerobic glycolysis; this is best seen with the more strongly

Table I. *Action of phenosafranine (formula I) on tissue metabolism.*

Medium	Conc. M	Phenosafranine			Control		
Rat brain cortex		Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
1. Phosph.	10^{-4}	$-8·3$	--	—	$-10·6$	—	—
	3×10^{-5}	$-9·2$					
	10^{-5}	$-11·3$					
2. Bicarb.	10^{-5}	$-12·8$	$+20·5$	--	$-12·6$	$+1·4$	—
	10^{-6}	$-10·0$	$+1·9$				
3. Bicarb. 2 hours:							
	10^{-5}	$-13·2$	$+9·7$	R.Q. 0·95	$-13·2$	$+1·7$	R.Q. 1·03
	dupl.	$-15·7$	$+12·4$	1·01	—	—	—
Chemical estimation of lactic acid:			0·71 mg.			0·07 mg.	
Manometric ditto.:			0·77 mg.			0·09 mg.	
4. Bicarb. in darkness:	10^{-5}	$-10·3$	$+10·5$	—	$-11·3$	$+0·3$	—
Mouse yolk-sac							
5. Bicarb.	5×10^{-5}	$-16·1$	$+29·8$	$+27·0$	$-12·3$	$+6·8$	$+32·0$
Jensen rat sarcoma							
6. Bicarb.	5×10^{-5}	90 min. $-9·3$	$+35·5$		$-11·3$	$+22·9$	-
	3×10^{-4}	$-9·7$	$+44·9$		$-10·8$	$+28·3$	-
Walker 256 carcinoma							
7. Phosph.	3×10^{-4}	$-9·3$	—	—	$-9·0$	—	--
	10^{-4}	$-7·7$					
	3×10^{-5}	$-8·4$					
Bicarb.	10^{-4}	—	$+36·2$	$+45·8$	--	$+24·5$	$+42·0$
8. JRS. Anaerobic glycolysis; phenosafranine tipped in after 40 min.							
				conc. 10^{-3}	3×10^{-4}	10^{-4}	Control
$\frac{Q_G^{N_2}\text{ (2nd. 40 min.)}}{Q_G^{N_2}\text{ (1st. 40 min.)}} \times 100$				61 %	81 %	86 %	84 %

glycolysing embryonic and tumour tissues (Exps. 5 and 7). Anaerobic glycolysis of these tissues is little affected (Exps. 5, 7 and 8) by concentrations of $3 \times 10^{-4} M$ or less.

Ashford & Dixon [1935] have shown that high concentrations of KCl increase respiration and aerobic glycolysis, and depress anaerobic glycolysis, of brain. Dickens & Greville [1935] showed that this effect was restricted to brain tissue. The action of phenosafranine differs from the KCl effect in several important respects: (1) the anaerobic glycolysis is not depressed by phenosafranine; (2) the action of phenosafranine is not restricted to brain; (3) the respiration, though sometimes a little increased, is not affected so much as by KCl; (4) the active concentration of phenosafranine is so low and its action on metabolism so selective that it is difficult to conceive of its action otherwise than as a specific anticatalyst of certain cell enzymes; whereas the action of KCl, or of disturbance of salt balance [Dickens & Greville, 1935], is an action on permeability and dispersion of cell colloids [Dickens & Greville, 1935; Dixon, 1936; Dixon & Holmes, 1935].

Mechanism of phenosafranine action: the effect of related compounds.

In seeking an explanation of this action, it appeared possible that the negative potential of phenosafranine (E_h at pH $7 = 0·252$ v.) might be responsible. Thus Lipmann [1934] considers that negative systems favour fermentative reactions and Bumm & Appel [1932] and Quastel & Wheatley [1932] have described a similar property of cysteine or reduced glutathione.

Consequently other negative dyes were tested (Table II) for their effects on respiration and glycolysis of rat brain cortex, but benzylviologen (E_h −0·359 v.) and even the chemically closely related compound neutral red (E_h −0·34 v.) were without action. Hence factors other than the negative potential are needed for the phenosafranine action.

Table II. *Other negative dyes.*

Medium	Conc.	With dye		Control	
Neutral red		Q_{O_2}	$Q_{G}^{O_2}$	Q_{O_2}	$Q_{G}^{O_2}$
Bicarb.	*ca.* 10^{-4}	−8·8	+1·7	−10·0	+1·4
Benzylviologen					
Bicarb.	10^{-5}	−13·0	+2·5	−11·1	+1·1
	10^{-4}	−8·9	+1·0		

Phenosafranine belongs to a group of dyes which are in use in photography as photosensitizers and desensitizers, phenosafranine itself being a powerful desensitizer. The precise mechanism of this photochemical action is still obscure, but it was decided to study the effects of members of this group on tissue metabolism. The formulae and reference numbers of the substances tested are shown on p. 1236, and their actions on metabolism of rat brain cortex are given in Table III.

Table III. *Sensitizers and desensitizers: action on metabolism of rat brain cortex.*

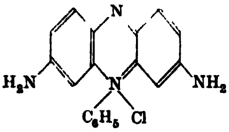
Formula No.	Name of dye	Sens. or desens.	Conc. *M*	Metabolism		
				Q_{O_2}	$Q_{G}^{O_2}$	
	None added	—	—	−10·5	+1·1	(average of controls)
I	Phenosafranine	D	(For data see Table I)			
II	*p*-Dimethylaminophenylazomethine-2-quinoline ethiodide	D	10^{-5}	−9·2	+17·5	
III	Pinacryptol yellow	D	*ca.* 10^{-5}	−9·0	+11·3	
IV	Pinacyanol	S	*ca.* 10^{-5}	−13·8	+16·8	
V	*p*-Dimethylaminobenzylidine-quinaldine ethiodide	S	10^{-5}	−8·5	+18·6	
VI	*p*-Dimethylaminobenzylidine-α-picoline methiodide	S	10^{-4}	−7·6	+32·0	(toxic)
VII	Ethyl red	S	10^{-5}	−12·0	+26·0	
VIII	*p*-Dimethylaminobenzylidine-β-napthoquinaldine ethiodide	S	10^{-5}	−9·2	+17·1	
	Derivatives of fluorescein:					
	Erythrosin B.	S	10^{-4}	−8·2	+3·4	
			10^{-5}	−8·2	+0·6	
	Eosin	S	10^{-4}	−8·1	+0·8	

It is evident from this table that representatives of both groups, sensitizers and desensitizers alike, may act in the same way as phenosafranine. Some of those tested quickly poisoned the respiration even in low concentration and were therefore discarded; others had hardly any action on respiration and in a few cases a slight increase was observed.

With all the active dyes however the aerobic glycolysis rose almost immediately on adding the dyes to a value approximately equal to that found in nitrogen.

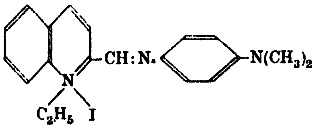
The concentrations needed are very low, usually $10^{-5}\,M$ as with phenosafranine. In higher concentration the dyes were found to be markedly poisonous, whilst in lower concentration ($10^{-6}\,M$) they were usually inactive.

Desensitizers.



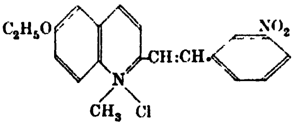
Phenosafranine

I.



p-Dimethylaminophenylazomethine-2-quinoline ethiodide

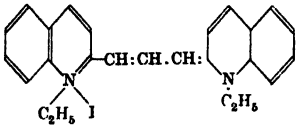
II.



Pinacryptol yellow

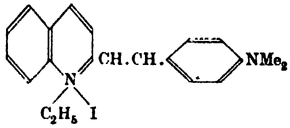
III.

Sensitizers.



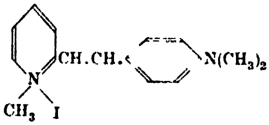
Pinacyanol

IV.



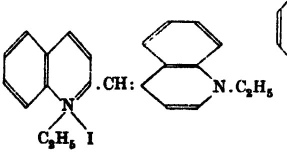
p-Dimethylaminobenzylidine-quinaldine ethiodide

V.



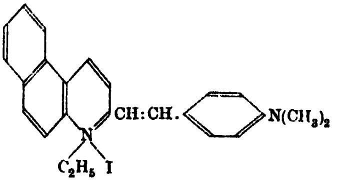
p-Dimethylaminobenzylidine-α-picoline methiodide

VI.



Ethyl red

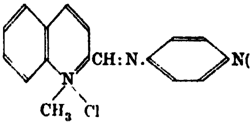
VII.



p-Dimethylaminobenzylidine-β-napthoquinaldine ethiodide

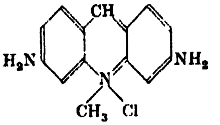
VIII.

Related compounds.



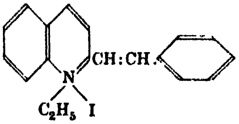
Quinanil (Cohen)

X.



Acriflavine

IX.



Benzylidinequinaldine ethiodide

XI.

Since both photosensitizers and desensitizers act similarly, it seems unlikely that the metabolic effect is connected with the photoactivity of the dyes, but more probably with a definite type of chemical constitution. This view is supported by the following facts: (*a*) the action of phenosafranine proceeds in as nearly complete darkness as is possible for manometric readings with the usual apparatus (Table I, Exp. 4); (*b*) eosin and erythrosin (also photosensitizers, but of completely different chemical constitution) have little or no effect on metabolism (Table III, Exps. 9 & 10); (*c*) chemically related compounds, some of which are not known to be sensitizers or desensitizers, act similarly to those already described (see Table IV).

Table IV. *Various related compounds: action on rat brain cortex.*

Formula No.	Substance added	Medium	Conc. *M*	Metabolism Q_{O_2}	Metabolism $Q_G^{O_2}$	Control Q_{O_2}	Control $Q_G^{O_2}$
				Group I			
IX.	Acriflavine	Phosph.	10^{-4}	−7·5 falling		−12·3	
			10^{-5}	−11·0			
		Bicarb.	10^{-5}	−10·5	+16·5	−9·1	+4·2
X.	Quinanil (*p*-Dimethylamino-phenylazomethine-2-quinoline methochloride)	Bicarb.	10^{-5}	−11·2	+13·0 (1st hour)	−9·8	+2·5
				8·9	+13·2 (2nd hour)	−10·6	+0·8
		Phosph.	10^{-5}	−11·5 (1st hour)			
				−9·5 (2nd hour)			
XI.	Benzylidinequinaldine ethiodide	Bicarb.	10^{-5}	−6·6	+17·6 $Q_G^{N_2}$ +19·5	−12·0	+1·9 $Q_G^{N_2}$ +19·2
			10^{-6}	−12·0	+3·8	−13·0	+0·7
				Group II.			
	β-Diethylamino-ethyl-1-aminoacridine hydrochloride [Clemo & Hook, 1936]	Bicarb.	10^{-5}	−7·4	+2·7	−7·8	+1·9
	Quinoline methiodide	Bicarb.	10^{-4}	−10·5	+3·5	−13·0	+2·0
	Quinaldine ethiodide	Bicarb.	10^{-5}	−12·0	+1·7		

It is possible from the data presented in Tables I–IV to draw some provisional inferences as to the general chemical constitution needed in substances of this group in order that they may specifically inhibit the Pasteur mechanism. All such substances are derivatives of a N-containing ring which may be phenazine (as in phenosafranine), quinoline or pyridine (as in the substances in Tables III and IV), or acridine (as in acriflavine, Table IV). In all the active derivatives of these parent substances described, one cyclic nitrogen is quaternary (phenochloride in phenosafranine, alkohalide in the other compounds). This combination alone is insufficient however, for quinoline methiodide and quinaldine ethiodide have hardly any action (Table IV) whilst phenazine methiodide and *NN'*-dimethyldihydrophenazine [Dickens, 1936] have a powerful catalytic type of action, instead of behaving as inhibitors or anticatalysts of the Pasteur mechanism. The introduction of the basic amino groups as in phenosafranine causes a complete alteration of properties of the *N*-substituted phenazine; the converse effect is seen in the presence of the acidic hydroxyl group in pyocyanine, which like phenazine methiodide depresses glycolysis. Similarly in phenosafranine loss of amino-groups or replacement by hydroxyl results in loss of desensitizing properties. The presence of the amino- (or substituted amino-) group may not be indispensable, however, in all cases; for benzylidenequinaldine ethiodide (Table IV) has a somewhat similar action to the other inhibitors, but this effect is here complicated by a rather strong poisoning effect on respiration. All active compounds contain a fairly long conjugated chain which, except in benzylidinequinaldine ethiodide and in pinacryptol yellow (III) which has a *m*-nitro-group, terminates in the amino-group. In this chain, as in the *p*-position to the cyclic N atom, $=CH-$ may be replaced by a nitrogen atom (e.g. compounds II and V).

Reduction of phenazine methiodide and of a derivative of quinoline ethiodide.

Phenazine methiodide (8·01 mg.) was ground with 4·0 ml. *N*/20 HCl until it was mostly dissolved and 1·5 ml. of the greenish yellow suspension were then transferred to a conical Warburg manometer vessel containing in 2 side bulbs 8 mg. Pt black in 0·5 ml. water. The vessel was filled with hydrogen and shaken nearly to absorption equilibrium in a bath at 37·5°. On tipping in the Pt, H_2 was taken up and the absorption was: 60 min. 160 μl.; 120 min. 190 μl.; 180 min. 206 μl., when it became exceedingly slow. Calculated for 1 mol. H_2 per mol. phenazine methiodide 208·5 μl. The solution became a very pale greenish colour and on standing in the air turned orange (cf. the oxidation of *N*-methyldihydrophenazine [Dickens, 1936] to a red aposafranone).

p-Dimethylaminophenylazomethine-2-quinoline ethiodide (II), 2·53 mg., dissolved with warming in 1·0 ml. water, were placed in the side bulbs of a Warburg manometer filled with H_2. The main part contained 10 mg. Pt black in 2 ml. water. H_2 uptake: 30 min. 186 μl. (colourless or very pale yellow); 200 min. 261 μl.; 240 min. 262 μl. (both exps. were corrected for a slow residual H_2 absorption by Pt). Calc. for $2H_2$ per mol. dye = 261 μl. H_2.

This exp. shows that the uptake of 1 mol. H_2 is accompanied by decoloration of the dye: this stage is reversible, shaking with air reoxidized the dye to give the original violet colour. With the entry of the second H_2 the colour is not restored by shaking with air.

The substances described in this paper therefore behave on mild reduction like the methiodide of nicotinic acid amide [Karrer & Warburg, 1936] and the coenzymes of dehydrogenation and fermentation.

Relationship of the inhibitors to cell catalysts.

As already pointed out, it is probable that these substances depend for their action on specific poisoning (or anticatalysis) of certain cell enzymes. Warburg [1926] has given a similar explanation of the inhibition of the Pasteur reaction by ethyl *iso*cyanide, which has a special affinity to certain heavy metal catalysts in "model" experiments.

The substances described in the present paper resemble ethyl *iso*cyanide in their effects on cell metabolism, but have an even higher affinity for the enzymes concerned in the Pasteur reaction, as is shown by their 100-fold lower active concentration. Whilst the nature of these enzymes remains unknown it is clear that the discussion of mechanism of their poisoning cannot be carried far: at the same time the use of specific inhibitors has often in the past provided a means of analysis of complicated enzyme actions. It is proposed to study the effects on various isolated enzyme systems of members of this group of substances with this end in view.

Until quite recently it was not known that substances of general chemical character resembling in any way that already described for the inhibitors were concerned in cell-catalysis. Warburg *et al.* [1936] have however shown that both the hydrogen-transporting coenzyme and the fermentation coenzyme contain as an integral part of the molecule nicotinic acid amide, and in a very recent paper [Warburg & Christian, 1936] it has been shown that the methiodide of the latter compound on reduction yields a substance of identical spectroscopic and reducing properties with the reduced active group of these coenzymes.

The chemical similarity of the substances described in this paper (e.g. the *N*-methylpyridine ring, with conjugated chain, of the substance VI) with Warburg's compound suggests that substances of this type may perhaps have a special affinity for enzymes, possibly those participating in the Pasteur mechanism. In the case of Warburg's coenzymes, an activation of the enzymes results from this combination: may it not be that with these inhibitors the same affinities cause the displacement of the active coenzyme by an inert anticatalyst, with consequent inactivation of the enzyme? Possibly the substances acting in an opposite direction (pyocyanine, phenazine methiodide, dimethyldihydrophenazine) may form similar, but active, complexes with the enzyme. Whether this is in fact the actual mechanism or not remains to be proved, but some such type of action is suggested by the specificity and the low active concentration of these substances.

Weil-Malherbe [1935], working in this laboratory, has found that simple dibasic acids (glutamic and maleic acids) strongly influence the Pasteur mechanism in certain tissues. It is probable that this is also primarily an interaction with tissue enzymes; its mechanism is being further examined.

Action of a sarcoma-producing quinoline derivative on metabolism.

Browning *et al.* [1933; 1936] have described the production of sarcoma in mice following a single injection of an aqueous solution of 2 (*p*-aminostyryl)-6-(*p*-acetamidobenzamido)quinoline methoacetate; i.e. of a compound closely related in chemical constitution to the 2-styrylquinoline derivatives found to inhibit the Pasteur mechanism as described above.

This resemblance caused us to study the effect of Browning's compound ("styryl 430") on tissue metabolism: the results are shown in Table V. The substance is freely soluble in water, but addition of salts to form Ringer solution caused precipitation of all but a small fraction: the freshly prepared semi-colloidal suspension thus provided was used for manometric experiments: a slow absorption by the tissue occurred as shown by the colour.

The most marked effect was observed with rat brain cortex. Corresponding with the slow absorption, the effect on metabolism is a gradual one, except anaerobically, when the anaerobic glycolysis is increased from the start. It is known that permeability is in some cases increased by anaerobic conditions [see Dickens & Greville, 1935]. In this tissue the respiration rose slowly (Table V), while the aerobic glycolysis similarly increased until it became about equal to the enhanced anaerobic glycolysis caused by the dye. This effect was shown by 7×10^{-5} *M* suspension, but was insignificant with a further 10-fold dilution, at least in a 4-hour experiment.

Guinea-pig brain cortex showed only the increased respiration, and this soon fell off when the same concentration as is active in rat brain was used. The reason for this difference is unknown. With the highly glycolysing growing tissues mouse yolk-sac and Jensen sarcoma of rat, the anaerobic glycolysis was increased at first in the former but not in the latter; increase of respiration was not observed and of aerobic glycolysis was very slight compared with rat brain.

Although we are unable to account for these differences, the effect with rat brain shows that under favourable conditions styryl 430 has a powerful effect in increasing respiration and glycolysis which resembles that of the other quaternary salts of 2-quinoline derivatives already described, together with some additional activities peculiar to itself and perhaps associated with the physical properties of this compound of high molecular weight. Whilst the effect on respiration is similar to the lyotropic action of K, Cs and Rb [Dickens & Greville, 1935] except that it is more slowly produced, the anaerobic glycolysis is much increased with the styryl compound whereas with KCl it falls. Brilliant cresyl blue [Dickens, 1936] caused a similar increase of $Q_{G}^{N_2}$, which however occurred in both

Table V. *Action of "styryl 430" (Browning).*

Concentration 7×10^{-5} *M* as suspension, unless otherwise stated.
Bicarbonate-glucose-Ringer solution *p*H 7·4. 37·5°.

		With dye			Control		
Tissue	Hours of Exp.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
Rat brain cortex	1	−12·1	+ 3·2	+31·2	−11·9	+ 1·6	+12·7
	2	−14·5	+ 6·1	+27·8	−12·3	+ 0·0	+13·2
	3	−20·4	+15·2	+24·6	−13·7	+ 0·5	+11·5
	4	−23·0	+24·7	+24·0	−13·7	+ 0·9	+12·5
(Four other exps. gave similar result with 7×10^{-5} *M* suspension.)							
(Same exp.: conc. 7×10^{-6} *M*: Q_{O_2}		(1) −13·6	(2) −13·5	(3) −14·7	(4) −14·2		
$Q_G^{N_2}$		+ 2·6	+ 1·6	+ 2·1	+ 2·6)		
Guinea-pig brain cortex	1	−17·3	+ 2·7	+17·6	−10·4	+ 2·5	+17·0
	2	−11·0	+ 4·1	+14·9	− 9·1	+ 4·2	+13·1
	3	− 4·2	+ 2·5	+16·5	− 8·3	+ 3·6	+13·7
(Another exp. gave similar result, $Q_G^{O_2}$ increasing from +2·6 to +2·8 only, after 5 hours' exp.)							
Mouse yolk-sac	1	−16·2	+ 5·5	+33·0	−18·5	+ 6·6	+25·4
	2	−13·4	+ 7·7	+26·0	−16·5	+ 7·2	+23·4
	3	− 9·8	+10·3	+21·6	−14·9	+ 8·6	+24·0
(Another exp. gave similar result.)							
Jensen rat sarcoma	1	−10·5	+24·0	+30·4	−10·5	+22·4	+30·4
	2	−10·9	+23·2	+29·0	− 9·6	+20·0	+32·0
	3	−11·5	+25·5	+33·6	−10·8	+23·6	+34·0
	4	− 8·7	+26·5	+31·8	− 8·5	+22·0	+36·0

rat and guinea-pig brain: 2:4-dinitrophenol [Dodds & Greville, 1934] increases respiration and glycolysis of tumour tissues, but in this material styryl 430 has little effect. Although alterations of permeability might be advanced to explain these phenomena, such an explanation is too vague to explain the different behaviours of anaerobic glycolysis towards KCl and styryl 430, for example, as well as the species differences reported above: the lack of sufficient data on permeability enables almost any problem of cell metabolism to receive a theoretical interpretation based on assumed alterations of permeability. The ingenious theories of Dixon [1936] appear to us to suffer from such a disadvantage.

In styryl 430 sarcoma production is seen to be associated with a special action on metabolism, whereby in favourable cases the anaerobic glycolysis of a "resting" tissue may be made to rise to the level found in rapidly growing embryonic and tumour tissues. The aerobic glycolysis increases more slowly, so that at one stage (e.g. 3rd hour, 1st Exp., Table V) the metabolism passes through a stage where it appears the same as for many tumours.

Unlike other cancer-producing agents which have been shown to have a direct influence on metabolism (arsenic, cyanide) styryl 430 induces experimental cancer readily in a high percentage of the animals used [Browning *et al.*, 1933; 1936], and at the same time it has the advantage over the carcinogenic hydrocarbons of being sufficiently soluble for metabolic effects to be produced *in vitro*. The rapidity of action on metabolism (at least with brain tissue) suggests a primary direct influence of the substance itself or of its reduced form rather than of some product formed from it by a slow reaction within the tissues. It is therefore peculiarly fitted for the study of the metabolic changes associated with induction of neoplastic growth.

The precipitation of styryl 430 by tissue fluids has been shown by Browning *et al.* [1936] to lead to the formation of a depot containing the dye at the site of injection, which remains for several months; this may determine the property of tumour induction following a single injection, which is seen with this substance. The other substances described in this paper are more soluble under these conditions, and as yet have not led to the production of malignant growths following a single injection. These animal experiments are being continued and extended.

SUMMARY.

The negative dyestuff phenosafranine inhibits the Pasteur mechanism in animal tissues, the active concentration being in the region of $10^{-5}\,M$. The negative potential is not alone sufficient to account for this action, since other negative dyes did not act similarly on metabolism. On the other hand a group of derivatives of pyridine, acridine and quinoline, containing pentavalent nitrogen and a conjugated chain terminating usually in a basic grouping, had essentially the same action on metabolism as phenosafranine, though some members were more poisonous.

The activity of this group is ascribed to an anticatalytic action on certain cell enzymes. Their general chemical similarity to the Warburg pyridine-body, the active group of the H-transporting and fermentation coenzymes, suggests that they may displace the coenzymes by preferential adsorption on the colloidal carrier, forming an inactive complex; but this remains to be proved.

The sarcoma-producing "styryl 430" of Browning, in concentration similar to phenosafranine increases respiration, aerobic and anaerobic glycolysis of rat brain very strongly; its effect on other tissues was less marked. These observations, and their possible relationship to experimental carcinogenesis, are briefly discussed.

To the following who have given valuable material and much helpful advice I would like to express my thanks: Prof. G. R. Clemo and Dr R. Raper (University of Durham), Dr Frances M. Hamer (Kodak Research Laboratories). I am specially indebted to Prof. C. H. Browning (Glasgow University) for kindly supplying a tested sample of purified styryl 430.

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CLXXVII. FURTHER EVIDENCE REGARDING THE ELIMINATION OF CERTAIN POLYCYCLIC HYDROCARBONS FROM THE ANIMAL BODY.

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(*Received 1 June 1936.*)

RECENT fluorescence studies [Peacock, 1936], in which fluorescent samples of bile were obtained after intravenous injection of colloidal solutions of certain polycyclic aromatic hydrocarbons (1:2-benzpyrene, methylcholanthrene and anthracene) into fowls, rabbits, guinea-pigs and mice, raised the question of the presence of these compounds in such samples of bile and in the rest of the animal.

Extraction of 1:2:5:6-dibenzanthracene had already been considered. In a previous communication [Chalmers, 1934] the quantity of dibenzanthracene in the breast muscles of fowls, at short intervals after local injection of the hydrocarbon dissolved in chicken fat or in egg-yolk fat, was determined spectrographically by absorption analysis of extracts in a fat solvent, and it was found that this hydrocarbon rapidly disappeared from the site of injection. Consideration of the possible excretion of unchanged 1:2:5:6-dibenzanthracene was deferred, as the extracts of excreta were unsuitable for absorption spectrography, owing to the presence of substances showing a general absorption in the ultraviolet. Although the methods of extraction were improved, the extracts were still unsuitable for absorption spectrum analysis. The contaminants however did not significantly mask the fluorescence spectrum of 1:2:5:6-dibenzanthracene, and subsequent analysis failed to reveal the presence of this substance in extracts of excreta. The fluorescence spectrum of 1:2:5:6-dibenzanthracene has already been described [Hieger, 1930]. Fluorescence methods were also used by Berenblum & Kendal [1936], who reported that no 1:2:5:6-dibenzanthracene could be detected in the excreta of mice injected intraperitoneally with the compound dissolved in lard or in colloidal solution. Lorenz & Shear [1936] have described a method of separating 1:2:5:6-dibenzanthracene from spectroscopically interfering substances in mouse extracts by the selective action of solvents on the unsaponifiable fraction. The colouring matter was then removed by chilling and absorption on Al_2O_3. In the preparation of 1:2:5:6-dibenzanthracene extracts for spectrography, as carried out by Berenblum & Kendal [1936], mice were killed and boiled under reflux condensers with alcoholic potash and subsequently extracted with benzene. The fluorescence of the benzene solution was then examined. In the extraction of 1:2:5:6-dibenzanthracene from excreta and from chick embryos, as will be seen, the ether extract after saponification with alcoholic potash required further treatment before fluorescence analysis was practicable. This further treatment involved the elimination of colouring matter by adsorption from a light petroleum solution of the material left after saponification and the removal of cholesterol. This method was applied to the other hydrocarbons under examination.

The evidence of chemical change of dibenzanthracene, after intramuscular injection, rendered it important to determine whether other polycyclic aromatic hydrocarbons (carcinogens and non-carcinogens) were altered in a similar way. At the same time, it was hoped to get an indication of the effect of the solvent on the rate of chemical change. Experiments on the production of sarcoma in fowls indicate that the solvent plays an important part in the induction of tumour growth [Peacock, 1935]. The results of these experiments hitherto may be summarized, as follows:

1:2:5:6-Dibenzanthracene in lard gave 16 tumours in 31 birds, in 1–4 years
1:2:5:6-Dibenzanthracene in egg-yolk fat gave 3 tumours in 12 birds, in 1 year
1:2:5:6-Dibenzanthracene in egg-yolk fat gave 0 tumours in 10 birds, in 1–3 years
1:2:5:6-Dibenzanthracene in chicken fat gave 0 tumours in 12 birds, in 2 years

Somewhat similar results were obtained by Watson [1935], who found that pinene tar, mixed with paraffin wax, produced sarcoma in 3 out of 12 rats, whilst the same tar, mixed with rat tissue extract, failed to produce tumours in a similar group of animals.

Methods of extraction.

Initial treatment. In early experiments, the following methods for extraction of fats, were tried: (1) treatment with acetone (a better solvent for 1:2:5:6-dibenzanthracene than alcohol) and (2) desiccation, followed by extraction with a fat solvent. In later experiments, however, treatment with strong caustic potash, as recommended by Berenblum & Kendal [1936], was adopted. This resulted in a considerable saving of time, as it provided an admirable preparation for the second stage.

Saponification. Care was taken to use specially purified reagents for this process and to reduce the saponification time to a minimum, as, otherwise, the action of heat on the alcoholic potash solution gave rise to the formation of resinous material which showed a blue fluorescence. The alcohol was purified by treatment with silver nitrate [Palmer, 1922]. After the initial treatment, as above, the extract only required dilution to 25–30% caustic potash and the addition of alcohol to be ready for saponification. The unsaponifiable material was extracted with ether in the usual way, one saponification usually being sufficient though, on occasion, a second treatment was required.

Precipitation with digitonin. The unsaponifiable fraction, which was obtained as a yellow solid, was dissolved in 95% alcohol and an excess of digitonin (1% in 90% alcohol) was added at the boiling-point. A good separation of the cholesterol was obtained by allowing the digitonide to precipitate out overnight, but satisfactory and quicker results were obtained by making use of the insolubility of the digitonide in water and acetone [Yasuda, 1931]. The ether extract of the diluted acetone solution was well washed with water to remove the excess of digitonin. Cholesterol was not removed except in the experiments with 1:2:5:6-dibenzanthracene embryos and excreta. The appreciable amount of cholesterol present in the chick embryos interfered with the examination of a final extract of less than 5 ml.

Treatment with adsorbents. Preliminary experiments were carried out with the pigment of egg-yolk fat. A certain amount of the colouring matter was eliminated along with the fat during the extraction of the unsaponifiable fraction. It was found that light petroleum was more effective than ether, alcohol, benzene or chloroform as a solvent from which to adsorb egg-yolk pigment. A number of adsorbents were tried: some adsorbed the hydrocarbon itself and were consequently valueless; of the others, fuller's earth was the most generally useful. A single treatment with fuller's earth, as a rule, removed most of the pigments from chick embryos and from excreta.

Excreta experiments.

1:2:5:6-Dibenzanthracene and 1:2-benzpyrene. Two fowls were injected intramuscularly with 1:2:5:6-dibenzanthracene (4 ml. of 0·2 % solution in chicken fat), and two with 1:2-benzpyrene (1 ml. and 4 ml. of 0·2 % solution in chicken fat), and the excreta were collected separately, over 2 weeks. According to previous work, if these compounds were eliminated unchanged, they should be detectable in the excreta during this period. The initial treatment in each case consisted of repeated extraction with acetone. 0·2 mg. of the corresponding hydrocarbon was added to one-half of the acetone extract, in each case, and thereafter the two fractions were treated alike. The residue, after evaporation of the acetone, was extracted by the method described above. The final extracts of excreta showed a slight blue fluorescence in the ultraviolet beam, but spectrographic examination failed to show the fluorescence bands of the appropriate hydrocarbon. Bands were however seen in the extracts to which the compound had been added as described.

Chick embryo experiments.

Intramuscular injection of 1:2:5:6-dibenzanthracene and 1:2-benzpyrene. Difficulties in the extraction of excreta from individual animals are obviated by the use of chick embryos, which may be regarded as a "closed" system. It was found best to open a window in the shell and membranes the day before injecting the embryo, rather than to attempt the whole procedure at one operation. In this way, it was possible to be sure that the embryo had survived the inevitable damage to the amniotic vessels and consequent slight haemorrhage. 15–18-day-old embryos were injected intramuscularly with 0·1 ml. of fatty or colloidal solution of the hydrocarbon under test, and the embryos were subsequently killed and extracted at short intervals. For purposes of this experiment, the chicks were not allowed to hatch out. It may be mentioned, however, that some chicks injected *in ovo* have been allowed to hatch out, and these showed no obvious abnormality.

1:2:5:6-Dibenzanthracene. The results of these injections were unsatisfactory, since the small quantity of the compound which could be injected into the embryo (0·1 ml. of 0·2 % solution in fat) did not give a sufficiently luminous fluorescence spectrum to permit of comparative tests of much accuracy, but it was found that the substance was altered within the embryo a few days after intramuscular injection.

1:2-Benzpyrene. The extracts of embryos injected with this substance gave a much brighter fluorescence than that obtained in corresponding experiments with 1:2:5:6-dibenzanthracene. The results of injections of 1:2-benzpyrene dissolved in egg-yolk fat are shown in Pl. II, fig. 1, from which it will be seen that most of the hydrocarbon was eliminated 90 hours after injection.

Intravenous injections of colloidal hydrocarbons.

Methods of preparing colloidal solutions of hydrocarbons have been recorded [Berenblum, 1932; Boyland, 1932; Boyland & Burrows, 1935]. The colloids used in the present experiments were prepared by precipitation of the hydrocarbon from solution in acetone, which was then removed by dialysis.

Technique of intravenous injection of embryo. 14–18-day-old embryos were selected for injection, since they are sufficiently large to be handled without receiving gross damage and yet are mobile within the shell. The egg was transferred from the incubator to a sterile dissecting chamber, without turning. The surface of the shell was swabbed with spirit-flavine (acriflavine, 1 part; water, 1000 parts; methylated spirit, 2000 parts), which dries rapidly. A window about 1 cm. square was cut in the shell with a sterile ampoule saw mounted in a handle, and the shell membrane was cut away with small sterile scissors, leaving the amniotic membrane exposed. Intravenous injection was sometimes made into an amniotic blood vessel, but, as a rule, the amniotic membrane was ruptured and the injection was made into the vitelline vein which is easily picked up as it lies on the yolk sac. The latter can be handled with small forceps, if necessary, to bring the vein into position for injection. No. 20 conjunctival needles were used for the injections and, although there was always a little haemorrhage, this was rarely severe. It was found advisable to wait for a few seconds after completing the injection before withdrawing the needle, to avoid possible loss of colloid. The window was then closed with sterile cellophane fixed to the shell with paraffin wax; this gave a free view of the embryo, which could thereafter be observed at frequent intervals without disturbance.

Elimination of 1:2-benzpyrene colloid after intravenous injection. The results of this experiment are illustrated in Pl. II, fig. 2, the explanation of which is given below in tabular form:

Time after injection	Colloid %	Volume injected ml.	Concentration of extract	Remarks on fluorescence spectrum
0	0·03	0·1	1·0	—
17 hours	0·03	0·1	1·0	No bands seen
0	—	—	0·025 (standard)	—
30 min.	0·003	0·3	1·0	-
20 min.	0·003	0·3	1·0	—
0	—	-	0·1 (standard)	—
6 hours	0·03	0·3	1·0	Intensity of bands greatly reduced
5 hours 30 min.	0·03	0·3	1·0	—
1 hour 30 min.	0·03	0·3	1·0	—
1 hour	0·03	0·3	1·0	—
40 min.	0·03	0·3	1·0	—
0	0·03	0·3	1·0 (standard)	—

Chick embryos killed shortly after intravenous injections of 0·3 ml. of 0·03 % 1:2-benzpyrene colloid showed highly fluorescent body fat when examined in the ultraviolet beam, this fluorescence being due to the dissolved 1:2-benzpyrene, as proved spectrographically.

It was noticed that the gall bladder of embryonic chicks showed no fluorescence similar to that shown by adult fowls and mammals. This appears to be due to the passive role of the embryonic gall bladder which, though filled with bile, does not seem to contract *in ovo*. This point is of some interest, and the view expressed is borne out by the following experiments in 1–3-day-old chicks.

Chick experiments. Incubator-hatched chicks are normally kept in the incubator for 2 or 3 days after hatching, during which time they have no access to food and are still nourished by their egg-yolk sacs which have retracted into the peritoneal cavity. Such chicks behave like embryonic chicks in that their bile is not rendered fluorescent by intravenous injection of 1:2-benzpyrene colloid. On the other hand, hen-hatched chicks, which had access to food, were found to behave like adult fowls in that their bile was rapidly rendered fluorescent by intravenous injection of the colloid. The passage of food from the gizzard to the duodenum apparently initiates the contraction of the gall bladder, which refills with fluorescent hepatic bile.

Mouse experiments.

To test the mechanism of hydrocarbon elimination on mammals comparable in weight with chicks, mice were injected in the tail vein with colloidal solutions of 1:2:5:6-dibenzanthracene, 1:2-benzpyrene, methylcholanthrene and anthracene. All except 1:2:5:6-dibenzanthracene mice showed the presence of a fluorescent substance in the bile 1–2 hours after injection. In making extracts of whole mice, initial treatment with caustic potash was followed by saponification. An almost colourless extract of the unsaponifiable fraction was obtained, and the fluorescent spectrum of this fraction was examined without further treatment.

Methylcholanthrene. The extract of an animal killed 3 hours after injection of 0·5 ml. of 0·01 % colloid did not show the fluorescence spectrum bands shown by the extract of a mouse killed immediately after injection.

Anthracene. Mice were injected intravenously with 0·5 ml. of 0·6 % colloidal solution and the animals killed at intervals after injection. The fluorescent spectra of the extracts of the whole mice are shown (Pl. II, fig. 3). It is just possible that the general fluorescence masks the anthracene spectrum in the early extracts, but in the 17-hour extract there is no evidence of the presence of the hydrocarbon.

Notes on fluorescence analysis.

The fluorescence spectra were recorded on a Hilger E 3 quartz spectrograph.[1] The source of ultraviolet rays was a Kelvin, Bottomley and Baird quartz mercury lamp, which was fitted with a Wood's glass filter, and the solutions were examined in a small flat-sided quartz cell fitted with a flat vita-glass cover to prevent loss of evaporation.

Pl. II, fig. 4 gives comparative spectra for the three carcinogens under test, and shows that the fluorescence of 1:2:5:6-dibenzanthracene is much less intense than that of methylcholanthrene or 1:2-benzpyrene.

In the course of the experiments carried out in this laboratory, the shift in the fluorescence bands of 1:2-benzpyrene with different solvents, described by Sannié [1936], has been observed, the bands in alcohol and light petroleum solutions being displaced about 50 Å. towards the ultraviolet region, relative to the bands in chloroform and benzene. Similar displacement in the fluorescence bands of 1:2:5:6-dibenzanthracene and methylcholanthrene in these solvents has also been noted. The importance of specifying the solvents used in such experiments is therefore obvious.

DISCUSSION.

The dosage of colloid, per body weight used in the case of small animals represented 5–10 times that employed in the case of large animals; comparable doses, in the case of fowls and rabbits, would have involved injection of about 50 ml. colloid. Equivalent dosage for small animals was therefore obtained by injecting a certain number with diluted colloid, which gave similar but less marked results. There seems to be no reason to doubt that essentially the same mechanism was involved in all the animals tested.

Benzpyrene, methylcholanthrene and anthracene appeared to be eliminated in the same way, but, as the fluorescence of benzpyrene and of its derivatives is much more brilliant and therefore more easily detected than that of the other hydrocarbons, most of the work has been done with this substance.

The course of events, after intravenous injection of 1:2-benzpyrene colloid, is substantially the same in rabbits, guinea-pigs, mice, fowls and chicks and may be summarized as follows:

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[1] Purchased with a grant from the British Cancer Campaign.

0–15 min. after injection. Fresh blood films examined by ultraviolet illumination show particles of yellow fluorescence in Brownian movement, the corpuscles appearing as dark non-fluorescent bodies. Naked eye examination shows that the animal's body fat is beginning to dissolve the benzpyrene, thereby acquiring a violet fluorescence.

15–30 min. after injection. Blood films show greatly reduced numbers of fluorescent particles which are larger and no longer show Brownian movement. They are independent of the blood cells. At this time, body fat shows violet fluorescence, proved spectrographically to be due to unaltered benzpyrene. Frozen sections of liver, examined by ultraviolet illumination, show lavender-coloured fluorescent droplets in the liver cells. (Morphologically similar droplets in controls show greenish fluorescence.)

30 min. to 2 hours after injection. Blood films show no fluorescence; body fat fluorescence diminishes; fluorescent bile appears in the gall bladder, reaching a maximum at from 1 to 2 hours. This fluorescence is due to a water-soluble derivative, or derivatives, which does not show fluorescent bands of benzpyrene.

2–6 hours after injection. Fluorescence of body fat disappears and fluorescence of bile diminishes and finally returns to normal (brownish green fluorescence).

1:2:5:6-Dibenzanthracene colloid disappears from the circulating blood like the other hydrocarbons investigated, but its subsequent fate has not been demonstrated. If it forms a bile-soluble derivative, this must be of feeble fluorescence or non-fluorescent.

Recently, most of our work has been done on mice and chicks, since the saving in costly materials and the ease of housing large numbers outweigh consideration of technical difficulties. As the intravenous injection of chick embryos does not seem to be a procedure in common use, a short description of the technique has been given above, but it may be mentioned here that chick embryos are quite easily handled and injected successfully after a little practice. 1–5-day-old chicks are much more difficult to inject, and light anaesthesia is required to prevent struggling.

As far as the colloids of 1:2-benzpyrene, methylcholanthrene and anthracene are concerned, the whole process, from the moment of intravenous injection to the time of final elimination by the liver, can be followed visually by virtue of the fluorescence of these hydrocarbons and their derivatives. Blood films for examination are examined fresh and unstained under a 4 mm. objective; no special lenses are required, as the ordinary optical glass transmits the near ultraviolet rays that penetrate the Wood's glass filter and are used to excite the fluorescence. The fluorescence of subcutaneous fat can be seen through the skin, particularly in the neck region of young chicks. Examination of samples of bile, in the case of fowls, was facilitated by the formation of biliary fistulae by preliminary cholecystostomy, from which bile could be withdrawn before and at frequent intervals after intravenous injection of the colloids. Fowls tolerate such fistulae well, but rabbits and guinea-pigs do not, and in these animals it was necessary to perform laparotomy for the collection of samples, at various time intervals after intravenous injection. The subsequent fate of the bile-soluble derivatives has not been satisfactorily determined, owing to the general nature of their fluorescence spectra. In the case of mice however it is possible to trace the fluorescent cystic bile in the duodenum and in gradually diminishing intensity in the small intestine. It is possible that destruction, or reabsorption, of these fluorescent derivatives occurs in the intestine, and that this explains their absence from the faeces.

Excretion in the bile is probably common to many substances in addition to those described, and it seems likely that any carcinogenic substance of the 1:2-benzanthracene type that might be formed in the body would be eliminated by this route. The problem of the mode of action of these carcinogens is somewhat simplified by the foregoing observations, for it seems clear that our efforts should now be concentrated on the early events of the latent period, rather than on that part of the cycle of events generally regarded as the earliest stage of malignancy, namely, the period of first demonstrable tumour growth. It is possible that the action of the chemical carcinogens is far more rapid than has been generally assumed, and that essential changes occur in the cell long before malignant multiplication commences. If this is so, it is possible that histological research may reveal hitherto unrecognized appearances characteristic of such early cytological changes. If, on the other hand, these carcinogens act as such and over a prolonged time, then the amounts required for carcinogenesis must be very minute and they must become fixed in some way at the site of injection so as to be protected from the process of elimination.

SUMMARY.

1. Methods of extraction of certain polycyclic hydrocarbons from chick embryos and from excreta are described.

2. Benzpyrene and dibenzanthracene have not been detected in the excreta of fowls injected intramuscularly with these substances in fatty solution.

3. Benzpyrene and dibenzanthracene are eliminated from chick embryos within a few days of intramuscular injection in fatty solution.

4. Benzpyrene is eliminated from chick embryos within a few hours of intravenous injection of colloidal solution.

5. Benzpyrene, methylcholanthrene and anthracene are eliminated from mice within a few hours of intravenous injection in colloidal solution.

6. The mechanism of elimination of benzpyrene, methylcholanthrene and anthracene, and the excretion of their fluorescent water-soluble derivatives in the bile and their recovery from the gall bladder, is described.

7. The absence of such fluorescent derivatives from the gall bladders of chick embryos and of 1–3-day-old incubator-hatched chicks previously injected intravenously with benzpyrene colloid is noted and explained.

We wish to acknowledge the technical assistance of Mr S. Breslin who also printed and arranged the spectra for publication, and of Mr C. Bern who assisted at the animal and chick embryo operations.

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Fig. I

Light petroleum extracts of embryos after intramuscular injection of 1:2-benzpyrene colloid

Time after injection hr.	min.	Concentration
0		0·1
0		0·2
0		0·5
0		1·0
90		1·0
47		1·0
24		1·0

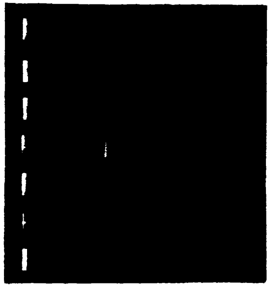
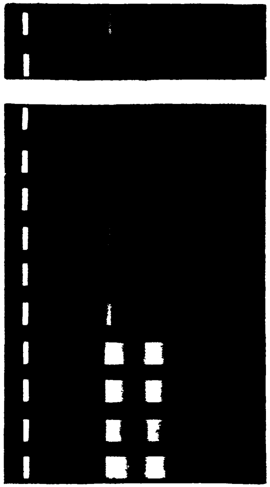


Fig. II

Alcohol extracts of embryos after intravenous injection of 1:2-benzpyrene colloid

Time after injection hr.	min.	Concentration
0		1·0
17		1·0
0		0·025
	30	1·0
	20	1·0
0		0·1
6		1·0
5	30	1·0
1	30	1·0
1		1·0
	40	1·0
0		1·0



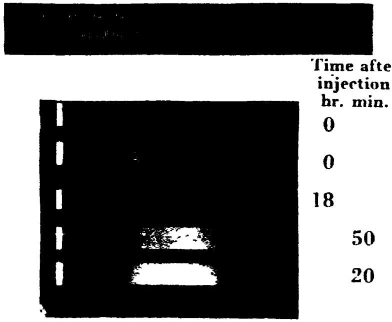


Fig. III

Alcohol extracts of mice after intravenous injection of anthracene colloid

Time after injection hr.	min.	Concentration
0		0·1
0		0·5
18		1·0
	50	1·0
	20	1·0

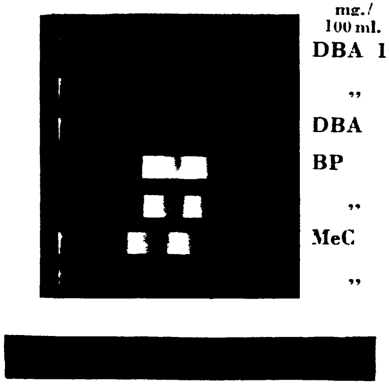


Fig. IV

Relative intensity of fluorescence in benzene

	mg./100 ml.	min. exposure
DBA	10	10
,,		20
DBA	1	20
BP	1	20
,,		10
MeC	1	20
,,		10

DBA	denotes	1:2:5:6-dibenzanthracene
BP	,,	1:2-benzpyrene
MeC	,,	methylcholanthrene

CLXXVIII. THE DISTRIBUTION OF VITAMIN A BETWEEN LIGHT PETROLEUM AND AQUEOUS METHYL ALCOHOL.

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The difference in behaviour on partition between light petroleum and 80–90 % aqueous methyl alcohol has for long provided a useful means of differentiation and separation of the hydrocarbon carotenoids from the corresponding hydroxylated compounds—the xanthophylls [Kraus, 1872; Borodin, 1883; Willstätter & Stoll, 1913]. The separation and extraction of carotenoids by this partition method can be controlled visually by the colour of the extract, but in analyses of biological materials containing carotenoids and vitamin A such as we have recently carried out on butter [Gillam *et al.* 1933], egg yolk [Gillam & Heilbron, 1935], and blood serum [Gillam & El Ridi, 1935], the question sometimes arises as to which phase contains the vitamin, which is, of course, colourless. In the analysis of butter it has usually been found that the vitamin A is divided between the petrol and alcohol phases rather irregularly, although Wolff *et al.* [1930] have shown that carotene and vitamin A can be separated by means of the phase test, the carotene going into the petrol phase whilst the vitamin goes into the alcohol phase. As however, the exact conditions for complete extraction were not specified and vitamin A has no colour by which its extraction can be followed visually, we have carried out a series of determinations of the partition coefficient of vitamin A between light petroleum and various accurately known concentrations of aqueous methyl alcohol, the conditions being strictly controlled.

EXPERIMENTAL.

Vitamin A. The material used throughout the experiments was a concentrate obtained by distillation of the sterol-free unsaponifiable matter of halibut liver oil [cf. Heilbron *et al.* 1932]. It was a pale amber-coloured viscous liquid having an intensity of absorption $E^{1\%}_{1\,\mathrm{cm.}}$ (328 mμ) = 1200 approx., i.e. it contained about 75 % of the vitamin.

Solvents. The methyl alcohol used (commercial pure) was further purified by refluxing with metallic sodium (20 g. in 2000 ml.) for 6 hours, followed by distillation, the first and last 100 ml. of distillate being rejected. The purified product was diluted with distilled water to the required density (by certified hydrometer) at 15°, the density-concentration values used being those quoted by Hodgman & Lange [1929]. The light petroleum was commercial material, B.P. 70–80°, redistilled.

Partition. 100 ml. of a solution of the vitamin A concentrate of suitable strength in light petroleum were transferrred to a dry 250 ml. stoppered bottle. 100 ml. of the aqueous methyl alcohol were added and the mixture shaken vigorously by hand at room temperature. The bottle and its contents were then immersed in a thermostat at 20° ± 0·5° and shaken occasionally during 1 hour. Portions of each phase were separated and their vitamin A contents determined.

Analysis of the two phases. Samples of each phase (2–5 ml.) were diluted with 25–100 ml. of ethyl alcohol (purified until completely transparent to ultra-violet rays) according to the amount of vitamin present, and the intensity of absorption of the solution, at 328 mμ, determined spectrophotometrically, using a Hilger E_3 quartz spectrograph and Spekker photometer. The concentrations of vitamin A in each phase were calculated from the intensity of absorption, on the basis of a value of $E^{1\%}_{1\,\mathrm{cm}} = 1600$ for the purest vitamin A, but the partition results—being relative—are independent of the absolute intensity of absorption of pure vitamin A.

The accuracy of the analytical method (photographic absorption spectrophotometry) is probably of the order of $\pm 2\,\%$.

Miscibility of light petroleum and aqueous methyl alcohol. The laws governing the distribution of solutes between two immiscible solvents are only strictly true when the solvents are perfectly immiscible. In the pair of liquids under discussion this ideal condition is apparently not realized but this does not affect the application of the phase test as a means of separation of, for example, carotene and lutein, or carotene and vitamin A, because the two phases are separated as such, irrespective of their composition. In the repeated extraction of a light petroleum solution with 90 % aqueous methyl alcohol the volume of light petroleum steadily diminishes by extraction into the alcohol phase.

The numerical results obtained in the partition experiments are recorded in Table I.

Extraction of vitamin A from one solvent by the other. Amongst other results of these determinations it is now possible to calculate how many extractions with a given concentration of aqueous methyl alcohol will be necessary to extract the whole of the vitamin A from a given solution in light petroleum. Thus, from the partition coefficient it follows that seven extractions of a light petroleum solution of vitamin A with 90 % methyl alcohol equal in volume to that of the light petroleum should be sufficient to remove all the vitamin A from the solution. We have tested this deduction experimentally and found the extraction of vitamin A to be almost quantitative.

When vitamin A is allowed to distribute itself freely between light petroleum and 90 % methyl alcohol the results show that the concentrations in each phase are approximately the same. This being so the reverse of the above procedure should also be true and seven extractions of the 90 % methyl alcohol solution with light petroleum should also remove practically the whole of the vitamin. This expectation has also been confirmed by experiment.

The effect of temperature on the distribution. All the determinations recorded were carried out at 20°, but in order to test the effect of variations of temperature, the 90 % methyl alcohol partition coefficients were also determined at 10 and 30°. The values were, within the experimental error, the same as those obtained at 20° (cf. Table I). It is therefore possible to work at any room temperature between 10 and 30° without affecting the results appreciably.

The effect of carotene. As it is frequently necessary to be able to separate carotene from vitamin A in biological material it was necessary to test whether small amounts of carotene affect the partition ratio. A standard solution of vitamin A in light petroleum was made up and pure β-carotene, approximately equal in weight to the vitamin A present, added. The mixture was extracted seven times with 90 % methyl alcohol, equal in volume to that of the light petroleum. The results showed that whilst the carotene remained in the petrol phase, 98 % of the vitamin A was recovered in the aqueous alcohol layers.

Table I. *Distribution of vitamin A between light petroleum and aqueous methyl alcohol (at 20°).*

Sp. gr. of methyl alcohol at 15° to water at 4°	Conc. of methyl alcohol % by wt.	Conc. of vitamin A in petrol phase (w/v %) C_1	Conc. of vitamin A in methyl alcohol phase (w/v %) C_2	Partition coefficient C_1/C_2	Volume changes‡ in phases of light petroleum and aqueous alcohol after partition. Initially 100 ml. + 100 ml. Final volumes		% of total vitamin A going into alcohol phase	
					Petrol	Alcohol	Assuming complete immiscibility	Allowing for volume change
0·810	95	0·0118	0·0177	0·67	—	—	59·3	—
		0·0159	0·0240	0·67	—	—	59·3	—
		0·0065	0·0102	0·64	—	—	61·0	—
		0·0138	0·0213	0·65	—	—	60·6	—
		—	—	**0·66**	77	123	60·0	70·5
0·824	90	0·0188	0·0194	0·97	—	—	50·8	—
		0·0177	0·0178	0·99	—	—	50·1	—
		*0·0203	0·0211	0·96		—	50·9	—
		*0·0206	0·0219	0·94	—	—	51·5	—
		0·0159	0·0160	0·99	—	—	50·1	—
		0·0175	0·0175	1·00	—	—	50·0	—
		†0·0191	0·0200	0·96	—	—	51·1	—
		†0·0188	0·0195	0·96	—	—	50·9	—
		0·0180	0·0190	0·95	—	—	51·3	—
		0·0177	0·0183	0·97	—	—	50·8	—
		—	—	**0·97**	90	110	50·8	55·7
0·838	85	0·0258	0·0181	1·43	—	—	41·2	—
		0·0250	0·0189	1·32	—	—	43·0	—
		0·0195	0·0138	1·42	—	—	41·4	—
		0·0195	0·0148	1·32	—	—	43·1	—
		0·0206	0·0151	1·36	—	—	42·4	—
		0·0188	0·0141	1·33	—	—	42·9	—
		—	—	**1·36**	94	106	42·4	45·3
0·850	80	0·0270	0·0101	2·67	—	—	27·2	—
		0·0270	0·0106	2·55	—	—	28·2	—
		0·0152	0·00583	2·60		—	27·7	—
		0·0270	0·00958	2·82	—	—	26·2	—
		0·0270	0·0096	2·81	—	—	26·2	—
		—	—	**2·69**	96	104	27·1	29·3
0·875	70	0·0359	0·00444	8·09	—	—	11·0	—
		0·0344	0·00437	7·87	—	—	11·3	—
		0·0362	0·00442	8·19	—	—	10·9	
		0·0352	0·0042	8·38	—	—	10·7	—
		—	—	**8·13**	97	103	11·0	11·5

* Both these sets of values were obtained at 30° (cf. text).

† These two sets of values were obtained at 10° (cf. text).

‡ These values were the means of actual volumes measured during the vitamin A distribution experiments but are not accurate to more than ±1 %.

The effect of sterols. In the biological material in which vitamin A occurs, there are always present relatively large quantities of sterols and the question therefore arises as to whether the partition coefficient of the vitamin is altered by the presence of sterols. In order to answer this question partition coefficients were determined as usual for light petroleum—90 % methyl alcohol, except that to the petrol solution of vitamin A was first added 1·2 % of cholesterol in one case and 2 % in another. The results are given in Table II.

Table II. *Effect of cholesterol on the partition coefficient of vitamin A between light petroleum and 90 °/₀ methyl alcohol.*

% of cholesterol in petrol solution (at beginning of exp.)	% of vitamin A in petrol phase C_1	% of vitamin A in alcohol phase C_2	C_1/C_2	% of total vitamin A in alcohol phase $\frac{C_2 \times 100}{(C_1+C_2)}$*	
2·0	0·0131	0·0085	1·54	39·3	40·1
2·0	0·0127	0·0088	1·44	40·9	
1·2	0·0188	0·0161	1·16	46·1	45·4
1·2	0·0180	0·0145	1·23	44·6	
0·0	0·0177	0·0183	0·97	50·8	

* Assuming complete immiscibility of the two phases (cf. Table I).

It is thus clear that the presence of sterols affects the partition coefficient considerably, in the direction of increasing the fraction of the vitamin remaining in the petrol phase. This is presumably due to the fact that the vitamin is extracted more effectively by a petrol solution of cholesterol than by petrol itself, for we know that in the partition the sterol goes preferentially into the petrol phase. Thus when use is made of the observed partition coefficients for work of a quantitative nature sterols should be removed as far as possible. It is probable that small amounts of sterol do not affect the partition seriously for it should be noted that in the above experiments the sterol: vitamin A ratio was of the order of 100–200 : 1.

SUMMARY.

In view of the usefulness of the Kraus distribution method (usually known as the "phase test") for separating carotenes from xanthophylls and the lack of accurate knowledge of the way vitamin A distributes itself under varying conditions when it occurs with carotenoids, a study of the partition coefficient of vitamin A has been made. The two solvents chosen have been those commonly used, i.e. light petroleum and aqueous methyl alcohol over the range 70–95 % alcohol. The results show that whilst with 90 % alcohol the vitamin distributes itself almost equally between the two phases, with 70 % alcohol the partition ratio is about 8 : 1 in favour of the light petroleum. It has been shown that it should require at least seven extractions of a light petroleum solution of vitamin A with an equal volume of 90 % methyl alcohol each time, to extract the whole of the vitamin from the light petroleum. This has been confirmed experimentally, as has also the conclusion that with this concentration of alcohol the extraction can be made as effectively from alcohol to petrol as in the opposite direction. The partition coefficient is unaffected by temperature changes between 10 and 30° but the presence of cholesterol alters it appreciably.

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CLXXIX. THE ABSORPTION SPECTRA OF STEROLS FROM VARIOUS NATURAL SOURCES WITH PARTICULAR REFERENCE TO ERGOSTEROL AND OTHER VITAMIN D PRECURSORS.

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THE fact that most of the animal and vegetable sterols can be rendered antirachitic by suitable irradiation with ultraviolet rays was, until recently, attributed to the presence of small quantities of ergosterol in the crude sterol. Support is lent to this view by the definite isolation of ergosterol from a number of its natural sources, e.g. fungi [Tanret, 1908], yeast [Windaus & Grosskopf, 1922], etc. In cases where it has not actually been isolated ergosterol has usually been detected by means of its characteristic absorption spectrum, consisting of a system of four narrow bands at 293·5, 281·5, 270 and 261 mμ in alcoholic solution [Pohl, 1926; Heilbron *et al.* 1927; Rosenheim & Webster, 1927; Windaus & Hess, 1927; Bills *et al.* 1928; Heilbron *et al.* 1928]. On the other hand, the preparation of 7-dehydrocholesterol, 22-dihydroergosterol [Windaus & Langer, 1933; Windaus *et al.* 1935], 7-dehydrocholestene and ergostatriene [Dimroth and Trautmann, 1936], all of which possess the same absorption spectrum as ergosterol, indicates that absorption spectra data alone cannot be accepted as sole proof of the presence of this compound, the more so as it has been demonstrated that 22-dihydroergosterol and 7-dehydrocholesterol also produce antirachitic products on irradiation [Windaus *et al.* 1935].

Moreover, cholesterol itself can apparently be rendered antirachitic [Bills *et al.* 1928; Koch *et al.* 1929; Waddell, 1934; Hathaway & Lobb, 1936], and the position caused by this acknowledged plurality of substances capable of activation is still further complicated by the slowly accumulating evidence tending to show that natural vitamin D is different from the artificially prepared calciferol [Steenbock *et al.* 1932; Ender, 1933].

With these points in mind and as part of a larger investigation we are endeavouring to isolate the selectively absorbing substance (or substances) which occurs in the sterol fraction derived from various animal products, the ultimate object being to ascertain whether the selective light absorption is in fact due to ergosterol or to some related body. As a necessary preliminary to this project, we have made an examination of the absorption spectra of a number of samples of crude and fractionated sterols, from diverse natural sources, with a view to finding the most suitable raw material for subsequent detailed examination of the absorbing compounds. The present paper records the absorption spectra data only (Table I).

The sterols derived from the lugworm (*Arenicola marina*), in particular, have been found to exhibit in addition to the ergosterol-like maxima, a triplet of bands at 346, 328 and 316 mμ. These maxima are closely comparable with those

Table I.

Source of sterol	Melting point ° C.	Ergosterol absorption bands	Gross value of $E^{1\%}_{1\,\text{cm.}}$ (281 mμ)	Estimated % of active substance as ergosterol	Comments and some relevant literature references
Ergosterol pure	162–164	293·5, 281·5, 271 (261) mμ	300	—	[Bacharach *et al.* 1933; Tanret, 1908]
22-Dihydroergosterol	152–153		(300)	—	Data by Windaus and Langer [1933; Windaus *et al.* 1935]
7-Dehydrocholesterol	142–143·5		(300)	—	
7-Dehydrocholestene	88–89		—	—	Data by Dimroth *et al.* [1936]
Ergostatriene	99–100		—	—	
Cholesterol pure	147	None	—	—	—
Cholesterol coml. "pure" *ex* fish liver oil	148	Bands plus much general absorption	0·33	Order 0·10	—
Cocksfoot grass	138	Very clear	2·43	0·81	[Gillam *et al.* 1933; Pollard, 1936]
Perennial rye grass	137	Very clear	4·45	1·48	
Butter (mixed sample)	—	Very ill-defined	0·71	Order 0·12	[Hentschel & Bachmann, 1930]
Colostrum butter	—	Clear	0·21	0·07	
Egg yolk	147	Very clear	0·66	0·22	[Bischoff, 1931]
Blood serum (cow)	144–145	Clear	0·14	0·15	—
Brain (cow)	146	Bands plus general absorption	0·11	Order 0·01	Solid unsap. = 22 % on dry wt.
Brain (sheep)	145–146	Bands plus general absorption	0·25	Order 0·04	Solid unsap. = 10·8 % on dry wt.
Brain (source unknown)	145–146	Selective absorption not detectable	0·07	<0·01	[Page, 1930]
Yeast	93–101	Very clear	59·1	19·7	Crude unsap. matter = 0·85 % dry wt. of yeast
Wheat germ oil	—	Fairly clear	2·4	0·8	[Drummond *et al.* 1935]
Halibut liver oil, 1	146	Very clear	0·30	0·10	—
Halibut liver oil, 2	—	Not present	—	None detectable	—
Japanese cod liver oil	146	Very clear	0·28	0·09	—
Whale liver oil	146	Clear	0·035	0·012	—
Cod roe (female)	147	Very weakly defined	3·49	Order 0·55	Solid unsap. = 8·0 % on dry wt.
Herring roe (female)	145	Bands plus general absorption	1·56	Order 0·35	Unsap. matter = 2·1 % on dry wt.
Plankton (*Munidia banffica*)	—	Plus general absorption	1·65	Order 0·35	Acetate (not free sterol) examined
Ragworm (*Nereis*)	148	Bands plus much general absorption	0·94	Order 0·20	—
Lugworm (*Arenicola marina*):					
(*a*)	135–136	Clear	35·9	12·0	—
(*b*)	143–144	Clear	27·2	9·0	—
(*c*)	—	Clear	13·9	4·6	—
Sea Mat (*Flustra securifrons*):					
(*a*)	142	Clear	10·3	3·43	Purified sterols (*a*)
(*b*)	132–135	Very clear	32·3	10·7	Crude sterols, another batch (*b*)
Seaweed (*Fucus vesiculosus*)	—	Rather ill-defined	0·23	0·08	Purified sterol
Oyster (*a*)	—	Very indefinite	10·7	Order 1·8	(*a*) Acetate, not free sterol examined
(*b*)	—	Very clear bands	16·5	5·5	(*b*) Crude sterol; another batch
"Ostreasteryl acetate" *ex Flustra securifrons*	—	Very clear	5·15	1·7	Oyster sterols [Bergmann, 1934]. Absorbing substance present as impurity
"Ostreasteryl acetate" *ex Pennatula quadrangularis*	142–143	Very clear	12·5	4·2	
Actinia equina	145	Very clear	15·0	5·0	Sea anemones [Heilbron *et al.* 1935; Klenk and Diebold, 1935]
Actinoloba dianthus (*a*)	134–138	Clear	11·5	3·8	
(*b*)	—	Clear	13·5	4·5	
(*c*)	—	Very clear	6·18	2·03	
(*d*)	—	Clear	31·9	10·6	
Anemonia sulcata	—	Clear	5·02	1·67	Fraction only of sterols

characteristic of the chromophoric grouping of dehydroergosterol (Heilbron *et al.* 1929]; the intensities of the bands in the lugworm sterols vary between $E^{1\%}_{1\,\text{cm}}$ 0·7 and 4·9 (equivalent to dehydroergosterol of the order 0·2–1·6 %). As however, identity of absorption maxima, in itself, is not conclusive proof of the presence of a particular compound we are endeavouring to isolate this absorbing substance also, with a view to examining its chemical and biological properties. Sterol fractions having absorption maxima similar to those of dehydroergosterol have also been obtained from wheat germ oil [Edisbury, 1933].

EXPERIMENTAL.

Preparation of the sterols. In the examination of the raw materials recorded in this work the main object has been to obtain the crude sterols with the minimum of manipulation since it has been found that the usual methods of purification tend to reduce the intensity of selective absorption of the "ergosterol" type. The sterols have, with few exceptions, been obtained by hot saponification of the raw biological material with aqueous or alcoholic potash followed by ether extraction, the examination generally being made on the solid portion of the unsaponifiable matter after one recrystallization from alcohol.

Determination of the absorption spectra. All the photometric measurements recorded were made on a Hilger E_3 Quartz spectrograph attached to a Spekker photometer, the light source being a tungsten steel spark for use when determining intensities. A hydrogen tube was also used occasionally to render more certain the detection of absorption bands when these were obscured by irrelevant absorption. The solvent used in all cases has been pure ethyl alcohol. No claim is made that the spectroscopic properties recorded for these gross sterol fractions are typical of the source from which they were derived, as only one or two samples of each kind have been examined. That two different samples of sterol from the same biological source may differ is shown by the variation between the two samples of halibut liver oil sterol and between the various samples of lugworm sterol (cf. Table I).

SUMMARY.

As part of a larger investigation into the apparent difference in properties between natural vitamin D and calciferol a survey of the absorption spectra of sterols from various animal sources has been made, with the ultimate idea of isolating the absorbing substances for subsequent examination. It has been found that certain marine animal sterols, notably those from lugworms (*Arenicola marina*), sea anemones and oysters, exhibit absorption bands identical with those of ergosterol and, calculated as such, indicate the presence of 5–12 % of the absorbing substance. The lugworm sterols exhibit, in addition, maxima at 346, 328 and 316 mμ, closely comparable with those of dehydroergosterol.

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CLXXX. THE POTENTIOMETRIC DETERMINATION OF POLYPEPTIDES AND AMINO-ACIDS.

II. THE FORMALDEHYDE TITRATION.

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(*Received 1 May 1936.*)

In a previous paper [Balson *et al.* 1935] a potentiometric examination was made of the Willstätter-Waldschmidt-Leitz titration. This titration was found to be of limited application to the study of proteins and their hydrolysis products owing to the low solubility of such substances in 90 % aqueous alcohol. We have accordingly made an examination of the formaldehyde titration of Sørensen, which as far as solubility is concerned, appears to be better suited to the examination of protein hydrolysates.

Previous work on the formaldehyde titration falls into two main divisions, firstly the isolation of compounds between formaldehyde and amino-acids, and secondly, the titration of amino-acids in the presence of formaldehyde with indicators or by electrometric methods.

Krause [1918], in a study of the reaction between glycine and formaldehyde, postulated the formation of a compound formed from two molecules of glycine and three of formaldehyde having the constitution

$$CHOH(CH_2.NH.CH_2.COOH)_2.$$

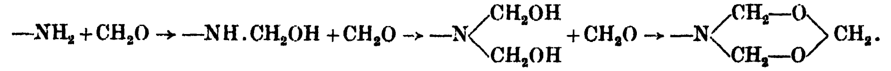
Bergmann *et al.* [1923] obtained compounds containing three molecules of formaldehyde with one amino-acid residue, and later [Bergmann & Ensslin, 1925] the triformylglycine obtained in this reaction was shown to be identical with the product of Krause. Bergmann's compounds were of the type $C_3H_6O_2.R$, one molecule of water being eliminated in the reaction, and they were readily converted into the corresponding methyleneimino-compounds of Sørensen [1908] and Schiff [1902] with loss of formaldehyde.

Harris [1924; 1929] and Birch & Harris [1930] have examined, both by colorimetric and potentiometric methods, the titration curves of amino-acids in formaldehyde solution. The treatment was not quantitative, but from the results Harris concluded that the action of the formaldehyde was to give the methyleneimino-acid of Sørensen, having a dissociation constant a thousand times as great as the parent acid. In the last paper of the series the effect of formaldehyde was used to prove the zwitterionic structure of the amino-acids. Richardson [1934] has made a comprehensive survey of the methods available for the determination of amino-N and -COOH and has discussed the significance of the results obtained in the light of the zwitterion hypothesis. The only quantitative work on the effect of formaldehyde on amino-acids is due to Levy [1933] and Tomiyama [1935]. Levy made a physico-chemical study of the equilibria involved and was able, assuming that formaldehyde reacts with the amino-acid anion, to give a quantitative interpretation of the reactions. With proline the reaction was found to produce only the binary compound, whereas with amino-acids both the mono- and di-formyl compounds were obtained. Tomiyama, restricting himself to the examination of the effect of small concentrations of formaldehyde (less than 0·2M), concluded that only one molecule reacts with the amino-acids glycine, alanine and proline. With such small concentrations, however, it is not surprising that the reaction does not proceed beyond the first stage. This restriction in formaldehyde concentration was applied to obviate possible errors in pH determination due to electrode failure and/or solvent change, but a consideration of the data presented in this paper shows that it is unlikely that any significant errors of such a nature are present. The equations developed describe quite adequately the data for formaldehyde concentrations up to about 5M. In the few cases where electrode failure was experienced the results were quite at variance with theoretical requirements, and under normal conditions the values for the reaction constants could be satisfactorily reproduced.

This work has been extended to include other amino-acids and imino-acids, dimethylglycine and a di- and tri-peptide. It has been found in agreement with Levy, that only the monoformyl compound is formed with the imino-acids proline and sarcosine, and also that both mono- and di-formyl compounds are formed with the amino-acids, but in addition, evidence is brought forward to show that a third molecule also enters into combination in the second case, and this behaviour has been found generally true for all the amino-acids examined. This is in agreement with the results of Bergmann. A di- and tri-peptide were also examined and found to give qualitatively the same results as the amino-acids. Dimethylglycine was found to be very slightly affected by formaldehyde, the effect being qualitatively the same as is obtained with boric acid. In view of this, and also because the results do not fit any mechanism involving combination with formaldehyde, it is concluded that no reaction takes place. The changes which do occur are probably in the main due to the weakly acidic function of the formaldehyde and in a lesser degree to solvent changes.

In view of these results, and taking into account the high reaction rate, it seems probable that the reaction takes place in stages. We therefore suggest that compound formation is dependent on the number of hydrogen atoms attached to the nitrogen. This will account for the addition of one molecule of formaldehyde to the imino-group. With the amino-group two molecules can be directly attached and the third molecule will most probably form a ring of the type of trioxymethylene. The following mechanism is suggested:

$$:NH + CH_2O = :N.CH_2OH$$



$$-NH_2 + CH_2O \rightarrow -NH.CH_2OH + CH_2O \rightarrow -N\begin{matrix}CH_2OH\\ CH_2OH\end{matrix} + CH_2O \rightarrow -N\begin{matrix}CH_2-O\\ CH_2-O\end{matrix}CH_2.$$

Tomiyama postulates the formation of a co-ordination compound between the formaldehyde and the amino-acid. Such a mechanism would imply that the deciding factor for combination is the presence of the lone pair of electrons on the nitrogen atom. On this basis one would expect only quantitative differences in behaviour between glycine, sarcosine and dimethylglycine. This, however, is not in agreement with our observations.

Birch and Harris have used the effect of formaldehyde on the titration curves of amino-acids to prove that they possess, in preponderating amount, the zwitterionic structure. It can be shown, however, that all the observations connected with this effect are not necessarily due to the presence of the zwitterion. Let us consider the titration of glycine with hydrochloric acid in the presence of formaldehyde (Fig. 3). Here we have a small but definite shift in the titration curve corresponding to a lowering of the apparent basicity caused presumably by reaction with formaldehyde. If the formaldehyde reacts with the —NH_2

group the effect on the apparent basic constant must of necessity be small since the apparent pK_b is 12; with a primary amine, on the other hand, the apparent basic constant is about 10^{-5} and by addition of formaldehyde this can be lowered to reach in the limit a value of 10^{-14}. Thus in the second case there can be a large possible effect on the titration curve with hydrochloric acid as Harris has shown.

The apparent acid constant of an amino-acid is about 10^{-10}, and the effect of formaldehyde is to increase this, so that a marked shift in the titration curve with sodium hydroxide towards a lower limit corresponding to the dissociation constant of a carboxylic acid can take place. The change in the acid constant can be quantitatively accounted for by assuming that the $—NH_2$ group reacts with formaldehyde. As is shown later, and as Levy has pointed out in this case, it is again immaterial whether the zwitterionic form, the normal form or the acid anion reacts. It will be seen therefore that the only effect of formaldehyde is to combine with the $—NH_2$ group to reduce its basic power, the $—NH_2$ group being either present as such or arising from the zwitterion.

It can be shown that it is immaterial as far as the results from the sodium hydroxide titration are concerned whether the formaldehyde attacks the anion, or the zwitterion or both. Thus if we consider the following equilibria:

$$\begin{array}{cccccc}
 & & (z) & & (a) & (h)\\
HA & \rightleftharpoons & Z^{\pm} & \underset{K_1}{\rightleftharpoons} & A' & +\,H^{+}\\
 & & + & & + & \\
 & & F & & F & \\
 & & A_z \uparrow\downarrow & & \uparrow\downarrow A_a & \\
 & & HAF & \rightleftharpoons & AF' & +\,H^{+}\\
 & & (b) & K_2 & (c) & (h)
\end{array}$$

Where z, h, a, F, b, c are the molar concentrations, K_1 and K_2 the dissociation constants, and A_z and A_a the association constants for the zwitterion and the anion respectively. We then have $K_1=ha/z$, $K_2=hc/b$, $A_z=b/zF$, $A_a=c/aF$, so that

$$K_1/K_2=A_z/A_a \qquad \text{......(1).}$$

Let us assume that the anions are attacked and that new acids are formed with dissociation constants different from the parent acids. If it is assumed that compounds containing up to three molecules of formaldehyde are formed, then the following equilibria are involved:

$$\begin{array}{llllll}
HA \rightleftharpoons & Z^{\pm} \overset{K_1}{\rightleftharpoons} & H^{+} + & A'\ (a) & \qquad & K_1=ha/z\\
 & (z) & (h) & + & & \\
 & & & F & & A_1=c/aF\\
 & & & \uparrow\downarrow A_1 & & \\
HAF & \overset{K_2}{\rightleftharpoons} & H^{+} + & AF'\ (c) & & K_2=ch/d\\
(d) & & & + & & \\
 & & & F & & A_2=e/cF\\
 & & & \uparrow\downarrow A_2 & & \\
HAF_2 & \overset{K_3}{\rightleftharpoons} & H^{+} + & AF_2'\ (e) & & K_3=eh/g\\
(g) & & & + & & \\
 & & & F & & A_3=m/eF\\
 & & & \uparrow\downarrow A_3 & & \\
HAF_3 & \overset{K_4}{\rightleftharpoons} & H^{+} + & AF_3'\ (m) & & K_4=mh/n\\
(n) & & & & &
\end{array}$$

Since the Henderson-Hasselbach equation describes the titration curves in the presence of formaldehyde we know that the apparent dissociation constant is given by:

$$K_0 = \mathrm{H}^+ \text{ (sum of all anions)/(all undissociated acids)},$$

i.e. $$K_0 = h\,(a+c+e+m)/(z+d+g+n).$$

This equation together with the above relations leads to the expression:

$$(K_0/K_1-1)+A_1F\,(K_0/K_2-1)+A_1A_2F\,(K_0/K_3-1)+A_1A_2A_3F\,(K_0/K_4-1)=0 \qquad \ldots\ldots(2).$$

Now if K_0/K_2, K_0/K_3 etc. are small compared with 1 this reduces to

$$K_0/K_1-1=A_1F+A_1A_2F^2+A_1A_2A_3F^3 \qquad \ldots\ldots(3),$$

which, apart from the last term, is Levy's expression. This expression is found to hold in practice and so the approximation is justified.

The value of K_0/K_1 can readily be obtained from the observed E.M.F. values as follows:

The amino-acid is approximately half neutralized with sodium hydroxide and titrated with formaldehyde. Before addition of formaldehyde is made the pK_a is given by:

$$pK_1 = p\mathrm{H} + \log\,(M-\mathrm{Na}^+)/\mathrm{Na}^+ \qquad (M = \text{molar conc. of amino-acid}).$$

This will also be true for the apparent pK_a values in the presence of formaldehyde.

Thus if E_1 = E.M.F. of hydrogen electrode before CH_2O addition,
E_0 = E.M.F. of hydrogen electrode at any CH_2O addition,

then at 25°: $$E_1/0{\cdot}0591 = p\mathrm{H}_1 \quad \text{and} \quad p\mathrm{H}_1 + C = pK_1,$$

where $C = \log\,(M-\mathrm{Na}^+)/\mathrm{Na}^+$ which is constant and independent of volume, and

$$E_0/0{\cdot}0591 = p\mathrm{H}_0 \quad \text{and} \quad p\mathrm{H}_0 + C = pK_0.$$

Therefore $$(E_1-E_0)/0{\cdot}0591 = p\mathrm{H}_1 - p\mathrm{H}_0 = pK_1 - pK_0 = \log K_0/K_1.$$

This does not involve the E.M.F. of the half cell.

The constants in equation (3) were obtained by graphical methods as follows:

Equation (3) is $$K_0/K_1-1=A_1F+A_1A_2F^2+A_1A_2A_3F^3=R.$$

Dividing through by F we obtain:

$$(K_0/K_1-1)/F = R/F = A_1 + A_1A_2F + A_1A_2A_3F^2 \qquad \ldots\ldots(4).$$

The plot of R/F against F yields a curve whose intercept is A_1 (see Fig. 1).

Taking the term A_1 to the left-hand side and dividing through by F we obtain:

$$(R/F - A_1)/F = A_1A_2 + A_1A_2A_3F \qquad \ldots\ldots(5).$$

This is a straight line intercept A_1A_2 slope $A_1A_2A_3$ (see Fig. 2).

The amino-acids give results which fit equation (3) and the various constants have been evaluated (Table I). Proline and sarcosine combining with only one molecule of formaldehyde give straight lines when equation (4) is applied, the slope being zero and the intercept A_1 (see Fig. 1). By means of equation (1), equation (3) may be converted into the corresponding equation which describes the attack on the zwitterion. Since however equation (1) does not involve the

Table I.

Amino-acid	α	β	γ
Glycine	160	500	69
Alanine	24	66	36·5
α-Aminobutyric acid	21	28	7·5
β-Phenylalanine	22	24·5	6·7
Valine	23	9·5	2·7
Leucine	17	32	2·7
Leucylglycine	22	35	9·4
Leucylglycylglycine	25	38	13·8
Proline	126	—	—
Sarcosine	320	—	—

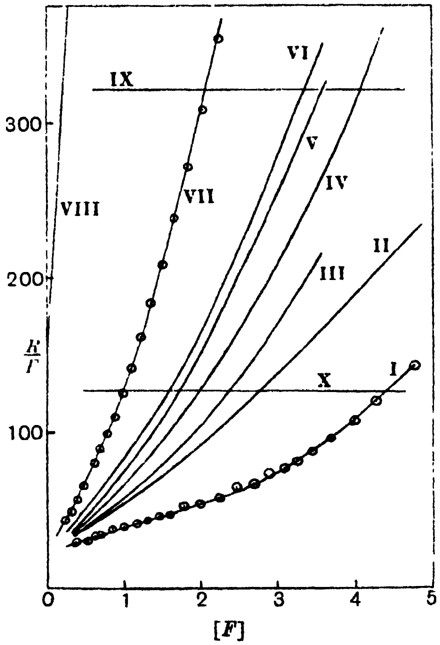


Fig. 1. Plot of CH_2O concentration against R/F (equation 4). Curve I, Valine. II, Leucine. III, α-Aminobutyric acid. IV, Leucylglycine. V, Leucylglycylglycine. VI, Glycine (on reduced scale R/F divided by 100). VII, Alanine. VIII, Glycine. IX, Sarcosine. X, Proline.

concentration of formaldehyde, then the new equation will be of the same order with respect to F as is (3), both being of the form:

$$K_0/K_1 - 1 = \alpha F + \beta F^2 + \gamma F^3.$$

The constants α, β, γ, may be determined by the methods of plotting already described. From the form of the curve no decision can be made as to whether the anion, the zwitterion or both react. These constants α, β, γ are thus composite association constants and from the data it is impossible to determine with which equilibria they are associated.

It would appear from the table of results that the constant for the addition of the first molecule of formaldehyde is practically unaltered with increase in chain length with the exception of glycine, whereas the second and third constants are markedly lowered. This is most noticeable in the cases of valine and leucine. It is interesting to note that chain increase caused by peptide formation results in the reverse effect in the case examined, the second and third constants showing an increase, as in the example leucine, leucylglycine and leucylglycylglycine.

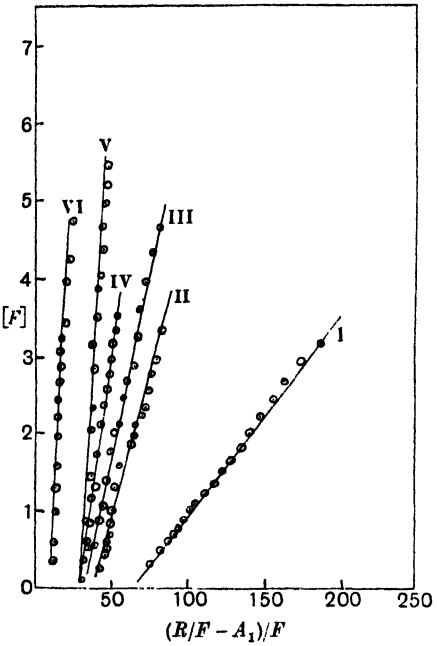


Fig. 2.

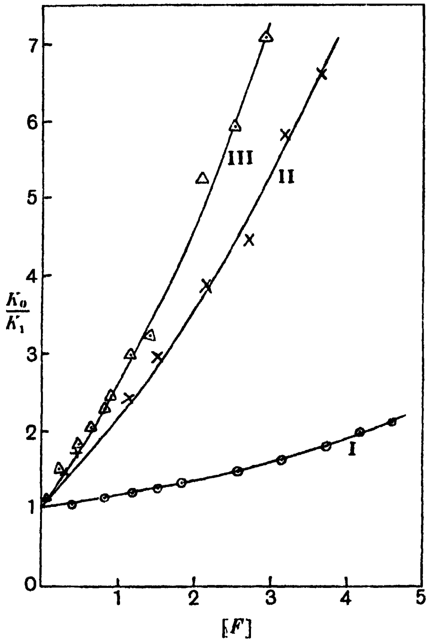


Fig. 3.

Fig. 2. Plot of CH_2O concentration against $(R/F - A_1)/F$ (equation 5). Curve I, Alanine. II, Leucylglycylglycine. III, Leucylglycine. IV, α-Aminobutyric acid. V, Leucine. VI, Valine.

Fig. 3. Plot of CH_2O concentration against K_0/K_1, where K_0 is apparent basic dissociation constant at zero CH_2O conc., and K_1 the value at conc. F. Curve I, Effect of CH_2O on glycine half-titrated with HCl. II, Effect of CH_2O on dimethylglycine half-titrated with NaOH. III, Effect of CH_2O on boric acid half-titrated with NaOH.

EXPERIMENTAL.

Glycine (A.R.) was used as standard, the other amino-acids being checked against this by electrometric titration. The formaldehyde was of A.R. purity and immediately before use was distilled over calcium carbonate, giving a distillate (*p*H 4·8) containing practically no formic acid. The distillate was filtered and immediately transferred to a sealed microburette, saturated with hydrogen and maintained in an atmosphere of hydrogen. Its strength (10–13 *M*) was accurately determined by the method of Romijn [1897]. All titrations were carried out in a thermostat at 25° using the titration vessel and hydrogen electrodes previously described.

SUMMARY.

1. The effect of formaldehyde on the titration curves of simple amino-acids and polypeptides has been quantitatively examined.

2. It is shown that mechanisms involving reaction between the formaldehyde and the amino-acid either in the form of the zwitterion, or the anion

or as both simultaneously yield results which fit the experimental data, so that it is impossible to decide in which form the amino-acid reacts.

3. All the primary amino-acids and polypeptides react with up to three molecules of formaldehyde, the secondary with one only, the tertiary being without action. A reaction mechanism is suggested.

4. The reaction constants for a series of amino-acids and two polypeptides have been evaluated.

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CLXXXI. A MICRO-METHOD FOR ACCURATE DETERMINATION OF D_2O IN WATER.

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DEUTERIUM oxide can be utilized as a tool for the study of certain chemical and biological problems, including the constitution of complex organic compounds *in vitro* and *in vivo*, and in a number of such cases it is essential to be able to determine very small quantities, available, say, from the combustion of tissue fragments or expensive compounds.

The method here described is a modification of one worked out by Barbour & Hamilton [1926] for specific gravity determination of blood and tissue fluids and also applied [Vogt & Hamilton, 1935] to D_2O. It consists essentially in measuring the rate at which a drop of water will sink through an immiscible fluid of slightly lower specific gravity. We have succeeded in increasing the accuracy to the sixth decimal place in specific gravity corresponding to 0·001 % D_2O in H_2O.

The important points in the method are:

(1) A very rigorous purification of the water to be tested, so as to exclude the presence of any substance save D_2O and H_2O.

(2) The production of drops of exactly the same volume by means of a special pipette.

(3) Temperature control of a water bath to within 0·001°.

(4) The measurement of time intervals of 15–30 sec. to within $\pm 0{\cdot}02$ sec.

(1) The water to be determined can sometimes be taken directly from an experiment, but more often it is obtained by distillation or even by combustion. When distilling off the water from organs we use a small oven which is heated to a constant temperature of 105° and evacuated by means of a filter pump connected up at intervals so as to keep the pressure below 10 mm. Hg. The water given off is condensed by means of solid CO_2 in alcohol giving a temperature of about $-70°$.

It is important that as much water as possible is driven off and condensed, because H_2O being slightly more volatile than D_2O a certain fractionation takes place.

The residues are pulverized and finally dried at about 108° in an electric oven or *in vacuo* below 100°, thereby losing from 2 to 10 % water.

Protein solutions are evaporated *in vacuo* from a flask to which minute quantities of octyl alcohol can be added (Fig. 1). Most of the water is condensed by means of ice and the rest by means of solid CO_2.

The D and H in organic combination are liberated by combustion. 0·5–2 g. dry powder is usually mixed with copper oxide-quartz and burned in a quartz tube in a current of dry air. That part of the tube containing the organic material is heated slowly and cautiously but the vapours must pass through copper oxide at 900°. The air is dried by $CaCl_2$ and P_2O_5 and supplied at the rate of about 50 ml. per minute.

In a few cases water distilled off, say from urine, must undergo an initial combustion to facilitate the final purification.

This is done by blowing the vapour through a small combustion tube with copper oxide heated to about 900°.

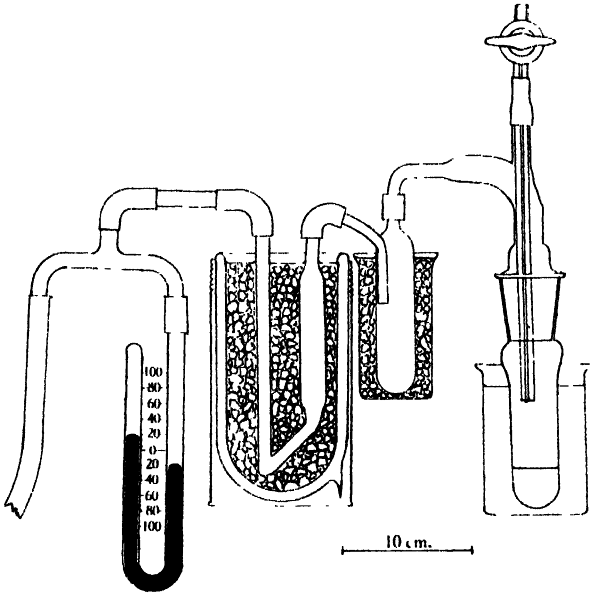

Fig. 1.

The routine purification which is sufficient in almost all cases is performed as follows:

The water sample of 0·5–2 ml. is heated in a sealed ampoule of 4 ml. capacity with about 25 mg. of permanganate and 1 mg. of sodium peroxide to about 150°, for not less than 1 hour. We use a small autoclave shown in Fig. 2 for the purpose and allow about 20 min. for raising the temperature, whilst the cooling to room temperature requires about 1 hour. Each ampoule is wrapped in filter-paper and a total number of 8 can be placed in a small porcelain dish. Almost all organic substances are destroyed by this treatment. It is of course essential that no substance should be present which can give rise to the liberation of D_2O.

The content of each ampoule is put into a pyrex distilling apparatus shown in Fig. 3 and distillations to dryness are repeated until the specific gravity becomes constant. Usually four distillations are required. Heating is done by means of a small flame which is moved by hand.

(2) The micro-pipette is shown in vertical section in Fig. 4. It is filled with mercury and the measuring device is the steel piston working from an adjustable stop (*A*) to a fixed one. The glass tube (*B*) is fixed in the nut (*C*) by de Khotinsky cement. The mercury column can be moved also by means of the screw in the top *D* and be made to fill the pipette completely. This device is used to wash out the pipette whenever a fresh fluid is to be measured. Consecutive deliveries of water from this pipette agree in weight at least to within 0·01–0·02 mg. The size used by us is 45 μl., but the pipette can be adjusted to any volume between 10 and 100 μl.

(3) The water-bath is a battery jar 480 mm. high, 240 mm. broad and 440 mm. long. The water is kept thoroughly mixed while it is cooled by tap water in a coil of lead tubing and heated by an electric bulb switched on and off by a thermostat arrangement. The variations in temperature should not exceed 0·001°. Once every few months the mercury contact should be cleaned with nitric acid to

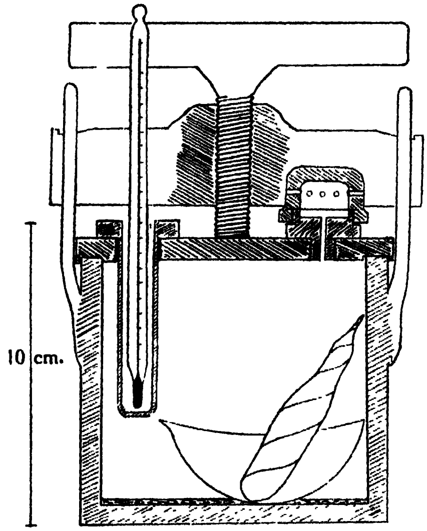

Fig. 2.

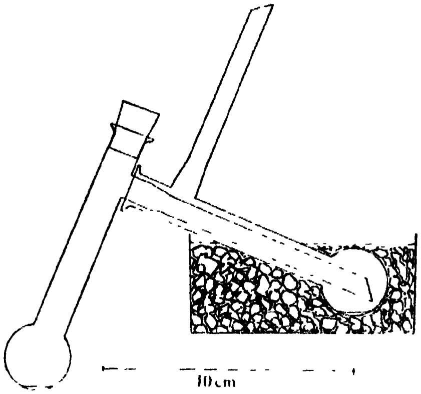

Fig. 3.

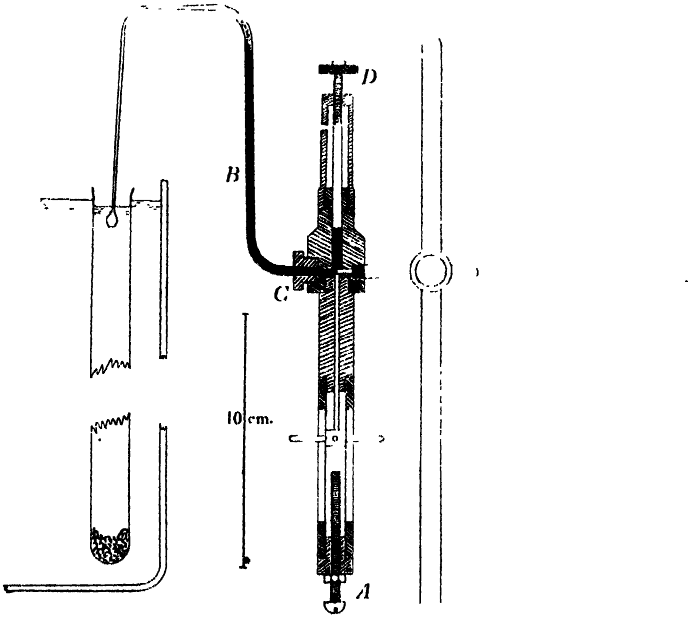

Fig. 4.

maintain maximum sensitivity. It is essential that the temperature is maintained slightly (about 1°) below that of the room. The water-bath should be sheltered against sunlight, strong radiant heat and draughts.

In the bath is a stand of 10 stoppered glass tubes 450 mm. long and 16 mm. in diameter. Each contains a different mixture of bromobenzol with xylene. In the lightest mixture drops of pure H_2O delivered by the pipette will fall 9 cm. in about 30 sec. The others are adjusted to give nearly the same rate of fall for 0·5, 1, 1·5, 2, 2·5 and 3 % D_2O respectively.

We have not found it possible to give definite instructions for the preparation of these mixtures. They are kept in stoppered bottles and adjusted by trial in the tubes with solutions of known specific gravity. When not quite right they are poured back into the bottle and a small amount of either bromobenzol or xylene is added. About 4 g. dry Na_2SO_4 are placed at the bottom of each tube to take up the water.

The measurement is made as follows.

The pipette is washed out and filled with the solution. The tip is placed just below the surface in the tube which is expected to correspond to the solution and the piston raised. If the drop is slightly warmer than the fluid the heat given off will not cause any disturbance by convection further down whilst even a slight difference in the opposite direction completely spoils the determinations. The drop is released by lifting the tip just clear of the surface and allowed to fall through 18 cm. before any measurement is made. We have arranged a stand with 3 microscopes with a magnification of 3–4 times at vertical distances of 90 mm. and use two stop-watches reading to 0·01 second. It is a very useful control on the temperature constancy and the absence of convection currents that the two readings on the same drop should agree within ± 0·02 sec.

(4) We have found it a little difficult to obtain stop-watches of sufficient accuracy and reliability and a chronograph would certainly be preferable. On the other hand the reading by means of microscopes is scarcely necessary. If the tubes were provided with circular marks at the appropriate distances reading by means of a simple lens could be made just as accurate.

All determinations are made as comparisons with solutions of known specific gravity, the falling rates of which are measured before and after that of the solution under test.

As standard solutions we use dilutions of concentrated D_2O with freshly distilled H_2O prepared by means of syringe pipettes [Krogh, 1935].

The calculations are made as shown in the following example:

Drop of 2·460 % D_2O in tube No. 6 falls 90 mm. in 28·30, 28·30, 28·28, 28·30 sec.—mean 28·295.

Drop of 2·952 % D_2O, 18·28, 18·26, 18·28, 18·30—mean 18·28.

The difference of 10·015 sec. corresponds to 492 units or 1 unit to 0·02036 sec. When the fluid under test takes 23·00 sec. to fall its concentration will be $2460+\frac{23{\cdot}00-18{\cdot}28}{0{\cdot}02036}=2645$ units.

Accuracy and sources of error.

The really dangerous source of error lies in the purification of the water samples for determination. The standard dilutions should be redistilled at least once a week, and whenever there is any doubt the experimental solutions are purified over again. To facilitate this samples should not be too small, less than 1 ml. being inconvenient. It is often necessary to dilute water obtained by combustion to obtain a sufficient quantity and the acuracy is of course reduced accordingly.

One important point is that D_2O solutions will enter into exchange with water vapour. They must therefore be carefully protected. When they are distilled in the way here recommended we have observed no change in standard water kept for a year and redistilled repeatedly.

The primary standard solution has a specific gravity of 1·1049, 99·6 % D_2O as bought from the Norsk Hydro. We have compared the standard solutions originally prepared and determined by Hofer over a year ago and redistilled repeatedly with fresh dilutions made up by weight from the 99·6 % D_2O. Assuming these latter to be correct, the water assumed to be 496 was found to be 498 and the water 2975 found to be 2973.

The syringe pipettes used for making up the dilutions of the standard are accurate generally to 1 part in 10,000 and the errors in the dilutions are therefore negligible. The errors on the size of the drop as delivered by the automatic pipette are also, we believe, too small to have any influence, being of the order of 1 in 5000 or less. The main source of error in the determination proper is in the rate of fall. We have been unable to get beyond 0·02 sec. on a distance of 90 mm. The difference in time corresponding to about 500 units is about 10 sec. so that the error corresponds to about 1 unit. We do not know whether the variations are real and due to temperature variations in the tubes or whether they are caused mainly by a personal error in the observer or by imperfections in the stop-watches employed, but we suspect these latter to be mainly responsible.

We give the following examples of results obtained:

1. Dilutions of D_2O 492 units

	Calculated	Found
$9H_2O + 1D_2O$	49·2	49
$8H_2O + 2D_2O$	98·4	97
$7H_2O + 3D_2O$	147·6	147
$6H_2O + 4D_2O$	196·8	195
$5H_2O + 5D_2O$	246·0	248
$4H_2O + 6D_2O$	297·2	294
$3H_2O + 7D_2O$	344·4	341
$2H_2O + 8D_2O$	393·6	393
$1H_2O + 9D_2O$	442·8	440

There is in this case a positive systematic difference of 1·3 units and accidental variations of about ±2 units.

2. A similar series of dilutions of D_2O 2975 with D_2O 2483 gave

	Calculated	Found
2 × 2483 + 8 × 2975	2876·6	2875
4 × 2483 + 6 × 2975	2778·2	2780
6 × 2483 + 4 × 2975	2679·8	2679
8 × 2483 + 2 × 2975	2581·4	2581

3. A sample of egg albumin dissolved in about 3 % D_2O was evaporated in a vacuum. The main portion of distillate was found to have a concentration of 3076 units. The final 1 ml. gave 3081. The increase observed can be taken as due to fractionation.

4. The egg albumin (dried completely at 108°) was burned with dry air in a quartz tube. The water produced was determined after dilution.

On one dilution of 0·1824–1·186 g. the concentration was determined as 190·6 units or for the undiluted sample 1240 units.

A second combustion yielding 0·2066 g. diluted to 1·2103 gave 208·4 units or for the undiluted sample 1240 units.

5. Rat urine distilled off through a combustion tube and diluted gave 400 units or for the undiluted 2550 units. The same urine purified by combustion but not diluted gave 2560 units.

6. Water which had been in contact for hours with human skin proved exceptionally difficult to purify. One such sample carefully purified in the usual way gave 2895 units whilst the same sample after combustion gave 2894 units showing that in this case the normal treatment was sufficient.

Results on the exchange of hydrogen atoms between the water and the tissues of organisms will be published shortly in the *Skandinavisches Archiv für Physiologie*.

SUMMARY.

Methods are described for obtaining water samples of 0·2–2 ml. by distillation or combustion, for purifying such samples without contamination, for measuring with a precision micro-pipette drops of any size between 0·01 and 0·1 ml. and for determining the D_2O content of such drops by their rate of fall in an immiscible fluid.

The accuracy is 1–2 in the sixth decimal place of the specific gravity or about 0·001 % of D_2O.

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CLXXXII. THE EFFECT OF WEANING UPON THE EXCRETION OF CALCIUM IN THE URINE OF LACTATING RATS.

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(*Received 20 April 1936.*)

Goss & Schmidt [1930] observed a marked loss of calcium from the calcium stores of the body of the rat during lactation. They did not separate the urine and faeces in their work, nor did they study the effect of weaning upon the excretion of calcium. It has been shown histologically by Selye [1934] and Jeffers [1935] that practically all the milk disappears from the mammary gland of the rat within 6–8 days after weaning, whilst on the second day after weaning the glands are quite distended with milk. Maeder [1922] claims that the secretory activity of the mammary gland of the rat is not noticeably diminished until the third day after weaning. Weatherford [1929] observed a decrease in the secretory activity of the mammary gland after the tenth day of lactation. This corresponded to the peak of the growth period of the young and the eruption of teeth. Involution of the mammary gland occurred more readily after the fourteenth day of lactation.

The present investigation was undertaken to study the effect of weaning upon the excretion of calcium in the urine of rats at different stages of lactation.

Methods.

Adult female rats were obtained from the colony on the day of birth of the litter: the size of the litter was reduced to 6 in number. The young were allowed to suckle the mother for a definite number of days and then weaned. The mother was placed in a Hopkins metabolism cage and the excretion of calcium in the urine determined for a period of 12 days. Urine was collected over a 2-day period. The whole specimen of urine was ashed on a heated sand-bath in the presence of concentrated nitric acid. The ash was dissolved in dilute hydrochloric acid and made up to a definite volume and calcium was determined on a suitable aliquot by the micro-method of Clark & Collip [1925].

Results.

The results are shown in Table I. The values are based on the average excretion of 4 rats in each group. The first column shows the effect of parturition on the excretion of calcium in the urine. These rats were placed in the metabolism cages 6 days before delivery. It is seen that the excretion of calcium in the urine of these rats is very small, amounting to approximately 0·1 mg. per rat per day. No significant increase occurred as a result of parturition which took place on the sixth day. Furthermore, no increased excretion of calcium occurred in the urine of these rats as long as the mother was allowed to suckle the young. It was not feasible to follow the calcium excretion of these rats for a longer period, since the young were not satisfactorily looked after by the mother in the open metabolism cage without any nesting material. However, the excretion

Table I. *The effect of weaning upon the excretion of calcium in the urine of lactating rats.*

(Expressed in mg. per rat per day; average of 4 rats.)

Days	Before and after parturition	Weaned at birth of litter	Weaned after 5 days' lactation	Weaned after 10 days' lactation	Weaned after 15 days' lactation	Weaned after 20 days' lactation	Weaned after 25 days' lactation	Mammary glands removed, 10 days' lactation
2	0·12	0·45	0·56	2·39	2·48	0·55	0·53	0·13
4	0·07	0·35	0·71	6·74	3·47	1·69	0·88	0·04
6	0·03*	0·04	0·04	0·05	0·10	0·10	0·05	0·08
8	0·06	0·20	0·07	0·03	0·05	0·06	0·05	0·04
10	0·04	0·14	0·04	0·02	0·04	0·08	0·02	0·09
12	0·11	0·15	0·05	0·02	0·05	0·08	0·04	—

* Day of parturition.

of calcium in the urine of this group serves as a control for the other groups as it shows a low excretion of calcium in the urine during suckling.

In the second column the effect of weaning at the birth of the litter is shown. It is seen that the calcium excretion in the urine is three to five times that found in the control group during the first 4 days. After this time, the urine calcium is quite within the limits of that found normally. When the young are allowed to suck for 5 days and then weaned, as shown in the third column, a slightly higher value is found for the first 4 days. When lactation is continued for 10 or 15 days (columns 4 and 5) a very markedly increased excretion of calcium in the urine occurs. It is noted that the excretion is greater on the third and fourth days of weaning than on the first and second. Thereafter the calcium excretion is similar to the control values. When lactation is continued for 20–25 days before weaning (columns 6 and 7), the values are decidedly lower than those found on the tenth and fifteenth days of lactation. The last column shows the results of removing all the mammary tissue of rats which had been lactating for 10 days; no increase in calcium excretion in the urine was observed. This shows that the calcuria of weaning is due to resorption of the milk from the mammary gland.

A group of 4 rats were weaned after 10 days' lactation; on the third day of weaning the serum calcium showed a slight increase. The average value was 12·2 mg./100 ml., whilst the value for non-lactating female rats was 10·4 mg./100 ml.

DISCUSSION.

The calcuria observed upon weaning lactating rats is of interest in that the animals do not appear capable of immediately restoring the depleted calcium supplies. Goss & Schmidt [1930] found that they could markedly deplete the bones of calcium by repeated pregnancies in rats on a low-calcium diet. Similar results have been reported for cows. The calcuria observed under these circumstances resembles the lactosuria observed by Brock and Hubbard [1935] and others in human subjects during the last month of pregnancy and for a time after parturition. The markedly increased excretion of calcium after 10 days' lactation agrees with the observations of Weatherford [1929] in that this time appears to be the peak of the secretory activity of the mammary gland. Cox [1936] finds that he can obtain a greater yield of milk from rats which have been lactating for 12–14 days.

An attempt was made to prolong the period of increased calcium excretion by the administration of the anterior pituitary hormone "prolactin". However, this treatment neither prolonged the period of calcuria nor caused a greater increase in the calcium in the urine than was obtained normally in rats which had been lactating for 10 days. Selye *et al.* [1933] report that ovariectomy after 26 days' administration of the anterior pituitary-like hormone of the placenta initiates lactation in non-pregnant female rats. However, upon repetition of this experiment, no increase in excretion of calcium occurred although slight evidence of milk was seen in the glands histologically. This negative result is undoubtedly due to the fact that it is very difficult to stimulate the mammary glands of the rat to a condition comparable with that found after parturition.

SUMMARY.

The weaning of lactating rats causes an increased excretion of calcium in the urine which is maintained for 4 days. A gradual increase in the calcuria was found from parturition until the tenth day of lactation; after this the calcium excretion was diminished. Removal of all mammary tissue from rats lactating for 10 days resulted in no increase in excretion of calcium. It is concluded that the calcuria observed upon weaning lactating rats is due to the resorption of milk from the involuting mammary gland.

The author wishes to express his sincere appreciation of the kind interest shown by Prof. J. B. Collip during the course of this investigation.

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CLXXXIII. THE EFFECT OF PARATHYROID HORMONE UPON THE SERUM CALCIUM AND CALCIUM EXCRETION OF NORMAL AND ADRENALECTOMIZED RATS.

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(*Received 4 May 1936.*)

It has been shown by Pugsley [1932] that administration of parathyroid hormone to rats causes a marked increase in urinary calcium excretion. With daily injections, the peak of the excretion is reached on the 4th or 6th day; after this time a gradual decrease in the calcium excretion to the control level occurs. Morgan *et al.* [1934] claim that the gradual failure of response to parathyroid hormone is associated only with a low-calcium diet and low Ca : P ratio; such a diet was used in the above work. That diet is an important factor in the response obtained to parathyroid hormone has been shown by the work of Allardyce [1931] on dogs, and of Shelling *et al.* [1933] on rats.

Schour & Rogoff [1936] have reported that the changes in the teeth following adrenalectomy in the rat are similar to those observed after administration of parathyroid hormone. They suggest a possible interrelationship between the parathyroid glands and the adrenals. It has been shown by Rubin & Krick [1933] that the loss of certain inorganic ions including calcium contributes to the development of the symptoms which follow adrenalectomy in the rat, and that administration of these ions will prevent the onset of the symptoms. The various factors involved in adrenal insufficiency in the rat have been reviewed recently by Cleghorn *et al.* [1936] and are outside the scope of this paper.

A study has been made of the effect of parathyroid hormone upon the serum calcium and calcium excretion of normal and adrenalectomized rats maintained on a supplement of 1 % sodium chloride. Data are also included on the effect of parathyroid hormone on the calcium excretion and calcium balance of rats on an adequate calcium diet and a normal Ca : P ratio.

Methods.

The rats were kept in Hopkins's metabolism cages and the urine and faeces collected separately. The collections were made over a 2-day period and the results are expressed in mg. Ca/rat/day. The analytical methods used in handling the excreta were similar to those described by Pugsley [1932]. The diet had the following composition: 31·1 % cornmeal, 31·1 % wheat flour, 2·1 % wheat germ, 31·1 % Purina, 2·1 % brewer's yeast, 2·2 % cod liver oil and 0·3 % sodium chloride. It contained 0·544 % Ca and 0·508 % P.

The rats were adrenalectomized under ether anaesthesia. 1 % NaCl was given as drinking fluid 10 days before the operation and continued throughout the experimental study. No difficulty was encountered in keeping the rats alive with this treatment. Adult male rats 5–6 months old were used in this part of the work. Parathormone (Eli Lilly Company[1]) was used in all the experiments. The dosage is expressed in Collip units.

The effect of parathyroid hormone upon the excretion of calcium in the urine of normal and adrenalectomized rats.

The effect of 1 % NaCl upon the excretion of Ca in the urine of normal and adrenalectomized rats is shown in Fig. 1. The data for this chart are based on the average excretion of 10 rats in each group. The rats in the upper half of the chart were placed on the NaCl 8 days before the operation and the urinary excretion of Ca followed. It is seen that the excretion is approximately 5–6 times that of the control rats receiving water (see first 8 days in lower part of chart). No significant difference in the Ca excretion in the urine is shown after adrenalectomy. The effect of 5 units of parathyroid hormone per day upon the excretion of Ca in the urine of normal rats is shown in the lower part of the chart. Injections were started on the 8th day and continued throughout the experiment. The maximum excretion of calcium occurred on the 4th day of injection; after this the excretion gradually returned to the control level.

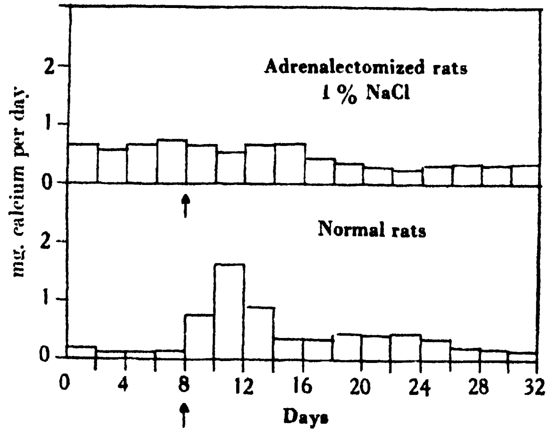

Fig. 1. Upper chart: The effect of 1 % NaCl as drinking fluid upon the excretion of Ca in the urine of normal and adrenalectomized rats. Adrenalectomized on the 8th day. Lower chart: The effect of 5 units of parathyroid hormone daily upon the excretion of Ca in the urine of normal rats receiving water as drinking fluid. Injections begun on the 8th day and continued throughout experiment.

Fig. 2 shows the effect of 5 units of parathyroid hormone daily upon the Ca excretion of normal rats (upper half of chart) receiving 1 % NaCl as drinking fluid. The rats were adrenalectomized on the 8th day and injections started on the 12th day. It is seen that the response is markedly increased (when compared with the normal rats shown in Fig. 1) and the adrenalectomized rats gave a greater response than the normal rats on 1 % NaCl. The additional effect on the Ca excretion cannot be attributed wholly to the NaCl, since over the 24-day period of injection the adrenalectomized rats excreted 22·0 mg. Ca and the normals 17·7 mg. Ca in the urine. This difference seems to indicate that adrenalectomized rats are more sensitive to the parathyroid hormone (Fig. 2). Over the same period, normal rats receiving 5 units of parathyroid hormone per day and with water as drinking fluid excreted 6·4 mg. Ca, while the adrenalectomized rats on NaCl but no parathyroid hormone excreted 5·5 mg. Ca (Fig. 1). Thus it is seen that the substitution of 1 % NaCl for water renders rats more sensitive to the parathyroid hormone, and adrenalectomy appears to enhance this effect. In a similar experiment, in which adrenalectomized rats received 10 units of parathyroid hormone daily and 1 % NaCl as drinking fluid, they excreted 37·7 mg. Ca in the urine over a period of 24 days. The maximum excretion occurred on the 4th day and amounted to 7·6 mg. Ca. This is a larger excretion of Ca in the urine than was ever obtained by the administration of 40 units of parathyroid hormone daily to normal rats with water as drinking fluid.

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[1] The authors are indebted to the Eli Lilly Company, Indianapolis, for supplying the parathormone for these experiments.

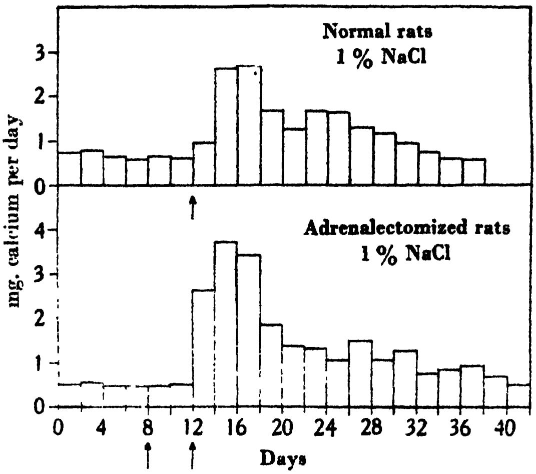

Fig. 2. Upper chart: The effect of 5 units of parathyroid hormone daily upon the excretion of Ca in the urine of normal rats receiving 1 % NaCl as drinking fluid. Note increased response as compared with Fig. 1. Lower chart: The effect of 5 units of parathyroid hormone daily upon the excretion of Ca in the urine of adrenalectomized rats receiving 1 % NaCl drinking fluid. Rats adrenalectomized on 8th day and injections begun on 12th day. Note greater response of adrenalectomized rats.

The effect of parathyroid hormone upon the serum calcium of normal and adrenalectomized rats.

This experiment was carried out similarly to the one described above. The hormone was injected daily and the blood obtained 12 hours after the injection by cutting the carotid artery and jugular vein and allowing the blood to flow into a centrifuge-tube. Ca was determined in the serum by the method of Clark & Collip [1925].

Table I, col. 2, shows the effect of 20 units of parathyroid hormone daily on the serum Ca of normal rats receiving water. It is seen that the maximum on the 4th day of injection is 14·2 mg./100 ml. Col. 3 shows the response of adrenalectomized rats receiving 1 % NaCl. The maximum here is 17·6 mg./100 ml. on the 3rd day of injection and 17·0 mg./100 ml. on the 4th day. The figures in the brackets refer to the number of determinations, and the average serum Ca of the group is taken. These rats, on the 3rd and 4th days of injection, showed symptoms of overdosage somewhat similar to those manifested in the dog. All of them showed hyperaemic areas in the stomach and caecum. In two cases there was

Table I. *The effect of daily injections of parathyroid hormone upon the serum calcium of normal and adrenalectomized rats.*

Expressed in mg./100 ml.

Days	Normal 20 units	Adrenalecto-mized + 1 % NaCl 20 units	Normal 1 % NaCl 5 units	Adrenalecto-mized + 1 % NaCl 5 units
Control	10·3 (40)	10·6 (9)	10·8 (4)	10·6 (4)
1	11·3 (3)	12·2 (2)	—	—
2	13·5 (3)	14·2 (3)	12·2 (4)	12·7 (4)
3	13·9 (3)	17·6 (5)	12·4 (4)	13·2 (4)
4	14·2 (4)	17·0 (5)	12·4 (4)	13·2 (4)

marked congestion of the alimentary tract and the presence of blood in stomach and intestines was noted. The condition of the stomach and intestines resembled that described by Collip [1926] as occurring in the dog when lethal amounts of parathyroid hormone were given. Normal rats receiving water tolerate 20 units of parathyroid hormone daily without any ill effects, and it is not until the dosage is raised to 40 units per day that ill effects occur, but even then the haemorrhagic condition of the stomach and intestines was not seen. Cols. 4 and 5 show the effect of 5 units of parathyroid hormone daily upon the serum Ca of normal and adrenalectomized rats receiving 1 % NaCl as drinking fluid. The serum Ca response is slightly greater in the adrenalectomized rats.

The effect of continued injections of parathyroid hormone upon the calcium excretion and calcium balance of normal rats.

The rats used in this experiment consisted of a litter of eight. They were weaned at 24 days of age, placed in the metabolism cages and the Ca excretion and balance followed for 104 days. 4 rats in the group were kept as controls and the other 4 were injected continuously with increasing amounts of the hormone as indicated in Fig. 3. Apart from the increase in urinary Ca which occurred during the first injections of the hormone, no essential difference can be detected between

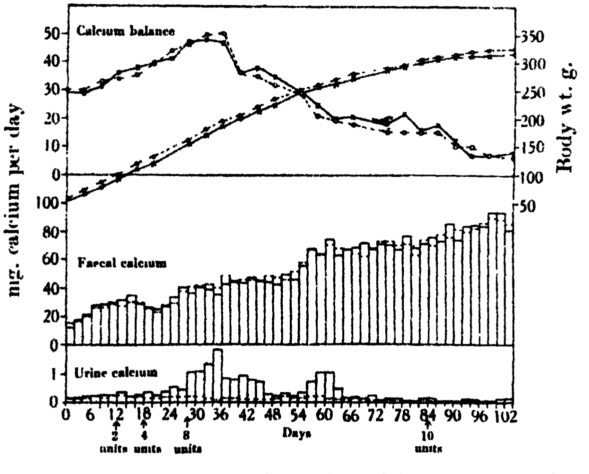

Fig. 3. The effect of continued injections of parathyroid hormone upon Ca excretion and Ca balance of young rats on an adequate Ca diet. Amount of hormone injected daily indicated by values below arrows. Continuous line = injected rats; broken line – control rats.

the two groups of rats. The Ca balance curves of the control rats (broken line) and the injected rats (solid line) follow each other very closely. The increase in urinary Ca due to the injections of the hormone does not alter the marked positive balance of these growing rats, since an increased excretion of 1·0–1·5 mg. Ca is insignificant in calculating a positive balance of 40–45 mg. No increased excretion of Ca in the faeces was found with this dosage of the hormone. An interesting relationship between the growth curves and the Ca balance curves is shown in this experiment. The rats have the greater positive balance of Ca during the period of rapid growth and as the growth curve flattens out the Ca balance becomes less positive. The decrease in the Ca balance is brought about by an increased excretion of Ca in the faeces.

These results show that rats become refractory to a given dose of parathyroid hormone when placed on an adequate Ca diet with a normal Ca : P ratio.

DISCUSSION.

The substitution of 1 % NaCl for water as drinking fluid and the resulting increased Ca excretion is possibly connected with the diuresis which takes place. This increased excretion of Ca is similar to that obtained by the administration of ammonium chloride to rats [Pugsley & Anderson, 1934]. The enhancement of the response to parathyroid hormone of adrenalectomized rats is of interest in view of the results of Schour & Rogoff [1936]. Our experiments indicate that Ca is more readily mobilized from the bones after adrenalectomy.

The results shown in Fig. 3 support the previous findings of Pugsley [1932] that daily injections of parathyroid hormone in the rat lead to an increased urinary excretion of Ca which gradually decreases to the control level in spite of continued injections. They are not in line with the suggestion of Morgan *et al.* [1934] that the gradual failure of response was due to the low-Ca diet which was used. These rats became refractory to the hormone, showing the same positive balance as the control rats. A low-Ca diet may be a contributing factor to the gradual decrease in response to continued injections of parathyroid hormone, since the rats used in these experiments on the high-Ca diet did not become resistant to the hormone as soon as the rats reported on the low-Ca diet. However, other factors discussed by Thomson & Collip [1932] play a role in the gradual decrease in response to continued injections of parathyroid hormone.

SUMMARY.

The substitution of 1 % NaCl for water as drinking fluid renders rats more sensitive to parathyroid hormone. Adrenalectomized rats maintained on 1 % NaCl show a greater increase in serum Ca and Ca excretion after injection of parathyroid hormone than normal rats under the same conditions. A haemorrhagic condition of the stomach and intestine was produced in adrenalectomized rats by the daily administration of 20 units of parathyroid hormone.

The Ca excretion and Ca balance of young rats on an adequate-Ca diet was followed. With continued injections of parathyroid hormone, these rats showed the typical increase in Ca excretion in the urine, which gradually decreased to the normal level.

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CLXXXIV. THE ROLE OF VITAMIN C IN ANIMALS RESISTANT TO SCURVY: EFFECTS OF INSULIN AND ADRENALINE.

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THE recent knowledge of the relation between vitamin C and the carbohydrate group of substances made it of interest to investigate any effect, on that vitamin, of substances such as insulin and adrenaline which have an influence on the storage, mobilization or utilization of carbohydrates in the animal body. Such an investigation necessitates the comparison of the amounts of the vitamin in the chief organs which contain it as well as the amounts excreted by animals before and after the administration of the substances.

It is well known that animals are divided into two classes, those affected by the deficiency of the vitamin in the diet and those which can withstand the deficiency. It is questionable which of the two classes will give more fruitful results under the conditions of the investigation. Among animals of the second class are rats, and it has been suggested by Parsons [1920], Parsons & Hutton [1924], Lepkovsky & Nelson [1924], and Parsons & Reynolds [1924] that the capacity of the rat for living without vitamin C may be due to the fact that these animals can synthesize it. Since it is aimed to include in this work a study of the possible influence of the substances above mentioned on the synthesis of vitamin C from precursors in the body, it was thought preferable to use animals which can synthesize the vitamin.

It is obvious that, unless the amounts of vitamin excreted and stored by such animals are controlled before the administration of the drugs, no reliable results can be obtained. At the same time an exact knowledge of the role of vitamin C in animals which are non-susceptible to scurvy when kept on a scorbutic diet is at present lacking. It was, therefore, planned to start the present investigation by the study of the storage and excretion of the vitamin in rats kept on an ordinary mixed diet and on a scorbutic diet for different periods, hoping at the same time to determine the best conditions for attaining instructive results about the influence of adrenaline and insulin on that vitamin.

EXPERIMENTAL PROCEDURE.

Certain organs and glands of animals, in general, contain vitamin C in higher concentration than most other tissues. The suprarenals and the liver have repeatedly been shown to be rich in the vitamin; further, the work of McCarrison [1919] shows that the suprarenals have a special physiological connexion with the vitamin. Although the concentration of the vitamin in the liver is smaller than in the suprarenals, yet, by virtue of the large bulk of the former its total content is far greater than that of the latter. The procedure adopted in this investigation is confined to the determination of the vitamin C content of the suprarenals, liver and the 24 hours' urine under different conditions. The method applied for the estimation of the vitamin in the organs and in the urine is the modification made by Emmerie & Van Eekelen [1934] of the methods of Birch *et al.* [1933] and Tillmans *et al.* [1932], which includes the removal of the substances interfering with the titration with 2:6-dichlorophenolindophenol by mercuric acetate followed by reducing the portion present in the oxidized form by hydrogen sulphide and titrating the total vitamin, in the reduced form, by means of a solution of the indicator standardized against a solution of pure ascorbic acid. The determination of the total vitamin, both oxidized and reduced, gives more accurate information, as it is possible that a portion of the reduced form undergoes oxidation during extraction from the organs or in the urine on standing, and, as has been shown by Tillmans *et al.* [1932] and by Hirst & Zilva [1933], the reversibly oxidized form of ascorbic acid has a similar activity to the reduced form.

Working with rats, however, presents certain difficulties. The suprarenals of a rat are too small to give, after the different manipulations involved in the above method, a sufficient volume of an extract of a reasonable concentration for the purpose of determination, but this difficulty was surmounted by using the four suprarenals obtained from 2 rats for each experiment and by modifying, by means of preliminary experiments, the proportions of the reagents to be used and the procedure to be adopted. All the rats used for the experiments were males taken from a stock which had been bred in the laboratory for many generations; the age difference did not exceed 7 days and the difference in weight between the animals did not exceed 10 g. They were kept and treated in exactly the same manner before being used for the experiments. After killing the animals by cutting the spinal cord, the four suprarenals were rapidly removed and prepared for the estimation of the vitamin.

In the case of the liver, the two livers obtained from the 2 rats were used for the extraction and determination of the vitamin.

The greater difficulty was met with in the case of the determination of the vitamin in the 24 hours' urine, as some factors interfere with the results. The first factor is the inexactness of the volume of 24 hours' urine due to varying amounts remaining in the bladder at the close of the period, but such an error is minimized by conducting a number of experiments and prolonging the time of observation under the same conditions whenever possible.The second factor is that the urine voided by rats may sometimes be alkaline or may turn so later, thus causing instability of the vitamin on standing. This difficulty was overcome by placing a measured volume of 20% acetic acid in the receivers and allowing for that volume in the calculations. The third factor is the change which the vitamin undergoes on standing. This factor is also minimized by acetic acid: but it has been found that ascorbic acid, even in acid medium, undergoes two sorts of change to a slight extent; one is reversible oxidation and the other is apparently destruction. Since the method of reduction before estimation was adopted, the oxidation did not matter. As regards the destruction, this was determined under the same conditions as were adopted for the collection of urine from the animals and was found not to exceed 14% at various air temperatures. It may be remarked that in the case of collection from rats the total destruction will be less than this, since the urine is voided at different intervals and therefore the time which elapses before the estimation is made is actually less than 24 hours for a considerable bulk of the urine. It has also been found that, even if the destruction amounts to 14 %, it does not seriously affect the interpretation of the results. Nevertheless, only limits of variations in a number of experiments, performed in the same manner and extended over long periods whenever possible, have been taken into consideration in the interpretation of the results.

Observations on vitamin C of the suprarenals and liver of rats.

A large number of rats weighing 220–230 g. and chosen as previously indicated were placed on the scurvy-producing diet recommended by Hassan & Basili [1932]. All rats kept on that diet gained in weight at the rate of 4–5 g. per rat per week. The vitamin contents of four suprarenals and two livers taken from 2 rats, which were killed at the same time and were chosen of exactly the same weight at the time of killing, were determined after different periods on the scorbutic diet. Similar rats to those used for each experiment but kept on ordinary mixed diet were also used for the determination of the vitamin contents of the same organs. The diet used for the latter animals was composed of equal amounts of bread, fresh clover and milk. The bread and clover were minced together and the milk was then added. This diet will be referred to in the text as ordinary diet. Table I gives the data obtained for the rats kept on the scorbutic diet for different periods.

It will be noticed that the total content of vitamin of the livers and of the suprarenals does not undergo any change up to 3 months, but, whilst the concentration of the vitamin, calculated in mg. per g., is almost constant in the livers, it shows considerable variations in the suprarenals. The gradual increase in the body weight of the rats, which amounts to 4–5 g. per rat per week during the 90 days on the scorbutic diet, has no effect on the vitamin content of the organs.

Table I.

Period on scorbutic diet days	Vitamin per g. suprarenals mg.	Total vitamin in 4 suprarenals mg.	Vitamin per g. liver mg.	Total vitamin in two livers mg.
3	4·60	0·23	0·24	3·54
6	3·92	0·22	0·24	3·66
9	2·27	0·24	0·22	3·39
13	2·18	0·20	0·23	3·52
18	2·27	0·23	0·23	3·72
23	2·36	0·22	0·22	3·52
28	2·65	0·21	0·24	3·72
35	2·87	0·20	0·23	3·70
45	3·61	0·22	0·20	3·37
55	3·59	0·20	0·19	3·29
65	4·60	0·23	0·20	3·56
75	4·58	0·21	0·19	3·64
90	4·22	0·23	0·21	3·66
Limits	2·18–4·60	0·20–0·24	0·19–0·24	3·29–3·72

The variations noticed in the concentration in the case of the suprarenals are ascribed to changes in the weights of these organs at different periods. It was observed that the suprarenals show a considerable increase in size and in weight between the 6th and 9th days up to the 28th day on the scorbutic diet. This is clearly indicated by comparing the concentrations of the vitamin in the suprarenals during the different periods (column 2, Table I). It may be remarked that this increase in the weight of the suprarenals is not in proportion to the body weight; this is evident from the comparison of the weights of the suprarenals during the different periods on a scorbutic diet with each other and with the weight of those of rats of the same weight but kept on the ordinary diet (columns 1 & 4, Table II). All weights are expressed in mg. per g. of body weight for the purpose of eliminating the factor which might be introduced by the latter in the comparison during the different periods, since the rats gradually increase in weight.

Table II.

Rats on scorbutic diet			Rats on ordinary diet		
Suprarenals per g. body weight mg.	Vitamin in 4 suprarenals mg.	Vitamin in two livers mg.	Suprarenals per g. body weight mg.	Vitamin in 4 suprarenals mg.	Vitamin in two livers mg.
0·116–0·124 (1–6 days)	—	—	—	—	—
0·132–0·223 (between 6 and 9 up to 28 days)	0·20–0·24	3·29–3·72	0·113–0·124	0·19–0·23	3·66–3·74
0·118–0·122 (28–90 days)	—	—	—	—	—

In view of the observation of McCarrison [1919] that the suprarenals of guinea-pigs in scurvy show an increase in size and in weight, the occurrence of this increase in the case of rats on a scorbutic diet was repeatedly confirmed. It extends only over a certain period and is greater about the beginning than at the close of the period. The nature of this increase is still under investigation, but it can be said at present that it does not appear to be a hypertrophy due to increase in the synthesis of the vitamin by these organs.

Table II gives a comparison of the total content of the vitamin in the suprarenals and livers of rats kept on a scorbutic diet for different periods with the vitamin contents of similar rats kept on the ordinary diet. The experiments show that the contents in the latter case are practically constant, regardless of the gradual increment of the weight of the animals, and that these contents do not alter in magnitude in rats kept on a scorbutic diet up to 90 days.

Vitamin C in the urine.

Rats of the same age and weight were placed on the ordinary diet. Every pair was placed in a Hopkins metabolism cage and the amount of food consumed by the pair in 24 hours was determined for some days. Every pair was then given an amount of the diet which would be completely consumed in 24 hours without causing inanition. After 1 week on the fixed amount of the diet, the urine was collected daily for the determination of the vitamin excretion in 24 hours. Five experiments were performed in this manner and the determinations were extended in each case for 15 days. After the close of this period, the diet was replaced by the scorbutic diet and the determinations were continued for 90 days. For the sake of brevity the results of the five experiments are represented in Fig. 1 where the limits of variation in the excretion of the vitamin per rat in 24 hours on the two diets for different periods are given.

The results show that in every case the excretion is variable. Taking the limits of variation into consideration, however, it is noticeable that, after the replacement of the ordinary diet by the scorbutic diet, there is a rapid fall in the vitamin C excretion which, in 48 hours, becomes less than half its previous value: thus, while the limits of variation in the excretion on the ordinary diet are from 0·85 to 1·22 mg. per rat per 24 hours, the limits from the 2nd to the 9th day on the scorbutic diet are from 0·34 to 0·55 mg. After the 9th day on the scorbutic diet there is a somewhat gradual increase in the excretion; thus, during the next 6 days the limits are from 0·55 to 0·825 mg. per rat per 24 hours. From the 16th to the 90th day the limits are from 0·60 to 1·08 mg., approaching on some occasions the higher limit of excretion reached in the case of the ordinary diet; the level of excretion during this period is generally lower than in the case of the ordinary diet.

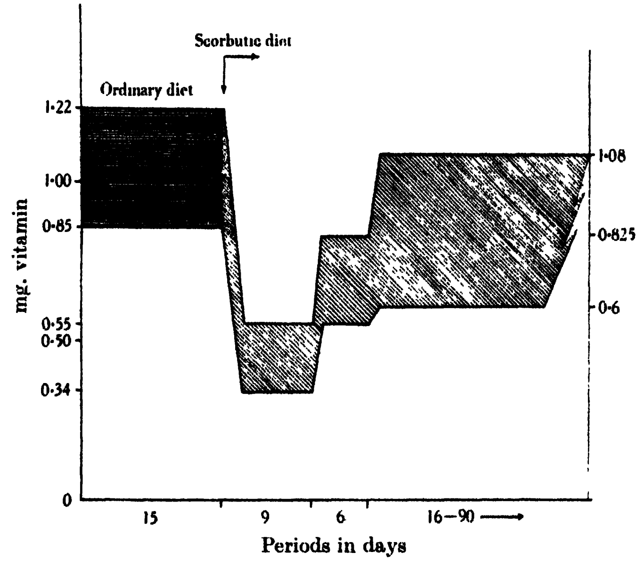

Fig. 1.

Effect of insulin on storage and excretion of vitamin C.

The above experiments show that the vitamin C content of the suprarenals and livers of rats is constant whether these animals are kept on ordinary mixed diet or on a scorbutic diet for different periods, whilst the excretion in the urine shows variations, the limits of which under the different circumstances are known in each case. It was, therefore, thought advisable to administer the insulin to rats which had been kept on a scorbutic diet for more than 16 days so that the variations in the excretion would have attained certain limits. All the rats used were kept on the scorbutic diet for 2½ months or more. The question also arose whether to give or to withhold the diet after the insulin injections. It is well known that insulin causes the animals to ingest more food if diet is allowed and hence a new factor, which could not previously be accounted for, would be introduced. It was, therefore, preferred to withhold the food during the 24 hours following the insulin injection. It then became necessary to determine the influence of withholding the diet for 24 hours on the vitamin content of the suprarenals and of the liver as well as on the amount excreted in the urine of rats previously kept on the scorbutic diet for the period indicated. The influence of insulin was also tried in some cases when diet was given.

A comparison of the vitamin contents of four suprarenals and two livers of 2 rats when starved for 24 hours with the usual content of these organs of two animals which were allowed to feed till the time of killing is given in Table III.

Table III.

Exp.	Starved for 24 hours: Total vitamin in 4 suprarenals mg.	Starved for 24 hours: Total vitamin in two livers mg.	Diet allowed: Total vitamin in 4 suprarenals mg.	Diet allowed: Total vitamin in two livers mg.
1	0·22	3·56	0·20	3·29
2	0·21	3·49	0·24	3·72

While starvation for 24 hours does not alter the vitamin C content of the suprarenals or of the livers it has a rather unexpected effect on the amount excreted in the 24 hours' urine. Thus while the amount excreted in 24 hours usually varies from 0·60 to 1·08 mg. per rat and never exceeds the higher limit, during starvation for 24 hours the limits of variations are from 1·10 to 1·35 mg. per rat, i.e. the excretion always rises (frequently to about 23 %) above the usual higher limit.

The results of the four experiments, graphically represented in Fig. 2, give strong support to this observation. 8 rats, previously kept on the scorbutic diet for about 2½ months, were placed in metabolism cages—one pair in each cage—and the scorbutic diet was continued. The vitamin excretion was determined and was found to be within the usual limits. The pairs were alternately deprived of the diet for 24 hours. The arrows indicate when the diet was stopped for 24 hours and each graph represents the excretion of one pair.

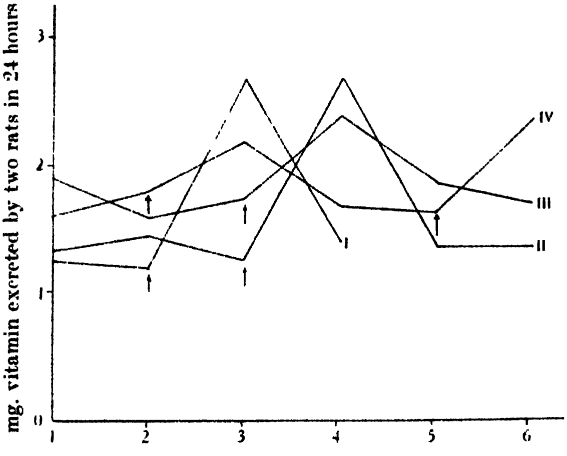

Fig. 2. ↑ Diet stopped for 24 hours.

It was next found that 4 units of insulin when injected into rats of 270–275 g. caused convulsions after about 10 or 11 hours and then death. Three units also caused similar effects, but a little later. The susceptibility to insulin was found to be the same whether the animals were previously kept on a scorbutic diet or on an ordinary diet. It was found that 2 units of insulin could be safely administered to rats of the above-mentioned weight when no diet was given.

Each pair of rats of the same age and weight, which had been kept on the scorbutic diet for about 2½ months, was placed in a metabolism cage. They were allowed to feed and the amount of the vitamin in the 24 hours' urine was determined daily in order to make sure that the excretion of these particular animals was within the usual limits. No abnormality was observed in any of the experiments. On the morning of one day 2 units of insulin were injected into each rat and no diet was given. After 23 hours, i.e. 1 hour before the rats were killed, 3 more units were injected into each rat. At the close of the 24 hours the rats were killed and the vitamin C contents of the suprarenals, liver and 24 hours' urine were determined. Since the contents of the organs were proved not to be altered by fasting for 24 hours, their content after the administration of insulin is compared with the usual content. The excretion in urine, however, is compared with the excretion of similar rats when fasted for 24 hours (Table IV).

Table IV.

Exp.	Vitamin in 4 suprarenals mg.		Vitamin in two livers mg.		Vitamin excreted per rat in 24 hours mg.	
	Insulin	No insulin	Insulin	No insulin	Fasting and insulin	Fasting and no insulin
1	0·20		5·46		1·20	
2	0·21		5·01		0·98	
3	0·20	0·20–0·24	5·32	3·29–3·72	1·15	1·10–1·35
4	0·22		5·43		1·25	
5	0·21		5·14		1·09	

The vitamin C content of the suprarenals does not change, on the other hand the content of the liver undergoes a remarkable increase, under the influence of insulin. This increase may possibly be due to an increased deposition of the vitamin in the liver at the expense of the content of other organs or to an increased synthesis by the liver, but, as it is not likely that the organs would contribute sufficient to cause this considerable rise in the content of the liver and at the same time keep the amount excreted in urine at a maximum, and further as it is found that there is no contribution by the suprarenal, one of the organs which contain the vitamin, the view of an increased synthesis is favoured.

As regards the vitamin C content of the urine, the limits of excretion after insulin administration and fasting for 24 hours are within the limits of excretion of animals simply fasted for 24 hours or just a little less.

The effect of insulin was also tried on rats while diet was given. Each pair of rats was placed in a metabolism cage and the scorbutic diet was provided. The amount of vitamin excreted by these particular animals in 24 hours before the administration of insulin was determined. No abnormality was again noticed. Each rat was then daily injected with 4 units of insulin for 4 consecutive days, the diet being still given. The vitamin excreted was determined daily and on the 5th day the rats were killed for the determination of the vitamin in the suprarenals and livers.

The vitamin contents of four suprarenals and two livers were 0·21–0·23 and 3·50–3·58 mg. respectively. The amount excreted per day during the 4 days of insulin injections was 1·00–1·08 mg. per rat. This indicates that there was no change in the content of either the suprarenals or liver but the amount excreted in 24 hours was always within the limits of excretion in the case of animals injected with insulin and fasted for 24 hours. Since the diet is given there is no explanation for the continued high level of excretion other than the assumption of an increased synthesis.

Effect of adrenaline on storage and excretion of vitamin C.

Beznák & Hariss [1934] tried the effect of adrenaline perfusion on the ascorbic acid content of the left suprarenals of cats, whilst the right glands were removed as normal controls. They found in three out of four cases that there was a decrease of about 20% of ascorbic acid in the perfused glands, but since in one case a decrease of the same order of magnitude was found in the right suprarenal, they came to the conclusion that perfusion with adrenaline caused no change in the ascorbic acid content. Their conclusion is contradictory to the finding of Euler [1933] that adrenaline injections cause the ascorbic acid to be liberated into the blood. Perfusion of the liver of guinea-pigs, previously saturated with ascorbic acid, also caused no change in the content of these organs.

In the present investigation, rats which had been kept on the scorbutic diet for about 2½ months were placed, in pairs, in metabolism cages. All rats were of the same age and of a weight 275–280 g. They were given the scorbutic diet and the amount of the vitamin was determined in the 24 hours' urine. No abnormality in the vitamin excretion by the particular animals used for the experiments was found. Each rat was then injected with 0·1 ml. of 1/1000 adrenaline hydrochloride solution and no diet was given for the next 24 hours. At the close of that period the pair of rats was killed and the vitamin C contents of the four suprarenals, of the two livers and of the 24 hours' urine collected were determined (Table V).

It can be concluded that adrenaline does not alter the vitamin C content of the suprarenals or of the liver. As regards the amount excreted in urine it can be seen that the administration of adrenaline when the diet is withheld for the 24 hours following the injection causes a remarkable fall below the limits of excretion of similar animals when simply fasted for 24 hours.

Table V.

Exp.	Vitamin in 4 suprarenals mg.		Vitamin in two livers mg.		Vitamin excreted per rat in 24 hours mg.	
	Adrenaline	No adrenaline	Adrenaline	No adrenaline	Fasting and adrenaline	Fasting and no adrenaline
1	0·23		3·50		0·38	
2	0·22		3·72		0·44	
3	0·20	0·20–0·24	3·69	3·29–3·72	0·60	1·10–1·35
4	0·21		3·67		0·52	
5	0·22		3·71		0·36	

Adrenaline is well known to cause glycogenolysis, hyperglycaemia and glycosuria, but it was repeatedly noticed that the urine of rats previously kept for 2½–3 months on the scorbutic diet does not contain sugar after the adrenaline injections. At first it was thought that either the dose was not sufficiently high to cause glycosuria or the preparations of adrenaline used were not sufficiently potent. These possibilities were eliminated by taking a number of rats of practically the same weight, 275–280 g., some of which had been previously kept on the scorbutic diet for the period above mentioned and some on the ordinary diet. Each rat was injected with 0·1 ml. of the same 1/1000 adrenaline hydrochloride solution, and no diet was given after the injections. Samples of 24 hours' urine were separately collected from each rat. It was again found that the urines of rats previously kept on the ordinary diet gave, with no exception, a strong reduction with Fehling's solution, whilst the urines of those kept on the scorbutic diet gave no reduction at all in the majority of cases, or a very slight reduction only in few cases. The occurrence of this very slight reduction may, at least partly, be ascribed to the possibility that some of the rats had a portion of the diet a shorter time before the injections than others, since it became much less frequent when the following procedure for feeding was adopted. On the morning of one day no diet was given to both batches of animals. The corresponding diets were given for 1 hour from 3 to 4 p.m. on the same day in order to insure simultaneous feeding of all the animals. On the second morning the rats were injected with adrenaline and were left with no food for the 24 hours following the injections. Under these conditions the urines of rats previously kept on the ordinary diet still showed considerable reduction of Fehling's solution after the adrenaline administration.

It was thought that a smaller store of carbohydrate in the body of animals previously kept on the scorbutic diet might be a factor in determining the difference in behaviour. It has been found, however, that the liver glycogen of both sets of animals was of the same order of magnitude at the close of the period usually preceding the adrenaline injections.

A difference which the two different diets might produce in the role of the adrenaline of the body was thought of as another possibility which might determine the difference between the behaviours of animals towards the injected adrenaline. If this is the case, the injection of equal amounts of adrenaline into animals which are otherwise under the same conditions but upon the different diets will cause a difference in the resultant total adrenaline which leads to a difference in the extent of glycogenolysis and of glycosuria in the two cases.

It may be remarked that the amount of adrenaline injected into rats in the two cases is about one and a half times as much as the normal total load of adrenaline of the two suprarenals and therefore a slight difference in the body adrenaline would not account for the marked glycosuria occurring in the case of animals previously kept on the ordinary diet and not in the others after the adrenaline injection. Nevertheless the adrenaline (mg. per g.) of the two suprarenals of rats kept on the scorbutic diet for 2½–3 months and of those kept on the ordinary diet was determined and was found to be the same; 1·890 mg. for those previously kept on the scorbutic diet and 1·892 mg. for those previously kept on the ordinary diet.

The following experiments prove beyond doubt that the difference in behaviour towards adrenaline is determined by the sort of diet given before the injections and is due to vitamin C in particular provided that the other experimental conditions are the same. Four groups of rats of the same age and weight were chosen. The first three were previously kept on the scorbutic diet for the period indicated, the fourth was kept on the ordinary diet. All continued to receive the respective diets. Group II was injected with 7 mg. of ascorbic acid per rat per day for 3 days before the adrenaline injections. Group III was treated like II but the ascorbic acid was added to the diet. On the day before the adrenaline injections the conditions for feeding already described were followed. All groups were injected with adrenaline hydrochloride (0·1 mg. per rat of 280 g.). The administration of ascorbic acid was stopped on the day adrenaline was injected. Examination of the 24 hours' urine following adrenaline injections showed no reduction of Fehling's solution in I, a marked reduction in IV, more reduction in III and a large reduction in II. Further, the reducing substances in terms of glucose in the 24 hours' urine per rat were determined by the method of Shaffer & Hartmann [1920] and the types of urinary osazones were examined under the different conditions (Table VI). This result, firstly, affords further evidence that no glycosuria is caused by adrenaline in the case of group I but a marked one in the other cases; secondly, that the increased reduction after adrenaline when ascorbic acid had been injected or given by mouth is due to glycosuria and not simply due to the ascorbic acid itself being excreted in the urine; thirdly, that ascorbic acid administration is not capable by itself of producing glycosuria.

Table VI.

Group	Glucose equivalent of reduction in urine (mg.) Before adrenaline	After adrenaline	Type of urinary osazone Before adrenaline	After adrenaline
I	9–12	10–14	N.M.	N.M.
II	11–16*	53–60	,,	N.M. and T.G.C. + + +
III	10–14*	37–44	,,	N.M. and T.G.C. + +
IV	11–15	24–29	,,	N.M. and T.G.C. +

* After ascorbic acid administration.

N.M. = normal mixture. T.G.C. = typical glucosazone crystals.

The results show that, under the same conditions, equal amounts of ascorbic acid given by mouth and by injections cause, after the administration of adrenaline, less glycosuria in the first than in the second case. This is probably due to destruction or loss of the vitamin in the alimentary canal.

With constant conditions of feeding before the adrenaline administration, the glycogenolysis and the resultant hyperglycaemia and glycosuria caused by adrenaline are, therefore, determined by the ratio of the dose of adrenaline to the body weight and by the degree of saturation of the body with vitamin C. The last statement will be referred to in the discussion.

The next question to investigate was whether the adrenaline, when injected into rats previously kept on the scorbutic diet for 2½–3 months, causes no mobilization of glycogen and hence no glycosuria or actually causes the former but at a lower rate than in the case of animals previously kept on the ordinary diet. For this purpose, the time at which the maximum rise in blood sugar usually takes place was determined when adrenaline was given in proportion to the body weight and the restrictions previously described were followed. It was found that, in the case of group IV, the maximum rise took place after 1 hour regardless of the body weight of the animals. Groups of rats were chosen and treated as described in the previous experiment. One rat of each group was used for determining the initial blood sugar level and the second received an adrenaline injection proportional to its body weight. All rats were slaughtered 1 hour after the injections and the blood sugar was determined. The mean of the results of a number of experiments is given in Table VII.

Table VII.

Diet	Initial blood sugar g. per 100 ml.	Blood sugar 1 hour after adrenaline g. per 100 ml.
Scorbutic only	0·102	0·210
Ordinary	0·104	0·247
Scorbutic *plus* ascorbic acid injections	0·112	0·268

While these results give further support to the previous observations they definitely show that adrenaline in all cases causes mobilization of the glycogen store of the body but the rate of mobilization is increased by increasing the saturation of the body with vitamin C.

DISCUSSION.

The discovery of the influence of vitamin C on the rate of mobilization of glycogen when adrenaline is administered is of great importance in making clear some of the results experimentally attained.

The saturation of the body by injecting vitamin C markedly increases the rate of glycogenolysis caused by adrenaline; further, the results show that this rate runs parallel with the degree of saturation of the body with that vitamin. Thus the highest rate for the mobilization of glycogen is attained by producing the highest saturation by means of injecting the vitamin, for 3 days, into rats previously kept on a diet deficient in that vitamin for 2½–3 months; a lower rate by adding the pure vitamin to the deficient diet of similar rats in similar amounts and for similar periods, when part of the amount given may be supposed to undergo destruction or loss; a still slightly lower rate by keeping similar rats on an ordinary mixed diet containing the vitamin; and the lowest rate when no vitamin is externally provided for the period above mentioned. This necessitates the assumption that, although the synthetic process is generally enhanced after the 9th day on the scorbutic diet and the level of excretion in urine also gradually rises and reaches certain higher limits from the 16th day onwards, yet the rats are to be regarded as in a less saturated condition than when they are kept on the ordinary diet. In fact, as has already been pointed out, the level of excretion of the vitamin in the urine from the 16th day onwards on the scorbutic diet is generally lower than the level of excretion when the animals are kept on the ordinary diet. These statements are not contradictory to the fact that these animals do not develop scurvy when kept on a scorbutic diet, since the amount of vitamin synthesized may be sufficient to ensure good health, while producing a relatively lower saturation than when the animals are kept on the ordinary diet. A similar finding, in the case of man, has been reported by Johnson and Zilva [1934], who found that it is possible to exist on diets containing sufficient vitamin C to insure freedom from scurvy even when the store of the vitamin in the body is maintained low.

It is indicated above that the rate of mobilization of glycogen under the influence of adrenaline runs parallel with the degree of saturation of the body with vitamin C. This rate can also be influenced by different factors. Since the adrenaline is given in proportion to the body weight and variable factors, such as the conditions of feeding before the administration of adrenaline and the age of rats, are controlled, whilst other factors, such as the adrenaline and glycogen contents of the suprarenals and liver respectively, are constant both in animals kept on the scorbutic diet for 2½–3 months and in those kept on the ordinary diet, it becomes obvious that the degree of saturation of the body with vitamin C is a direct factor in determining the rate of glycogenolysis. Against this statement must be set the fact that saturation of the body with ascorbic acid alone has been proved in the present work to produce no hyperglycaemia or glycosuria; but the rate of rise of blood sugar is determined by the rates of the mobilization of glycogen and of the utilization of the blood sugar and unless the latter is interfered with, as is done by injecting adrenaline, the effect of the degree of saturation of the body with the vitamin on the rate of rise of blood sugar will be rendered inconspicuous. This becomes more evident if we take into consideration the recent work of Strieck [1935], who found that vitamin C, administered in different manners, increases the oxygen consumption without affecting the respiratory quotient or, in other words, that the carbohydrates are included in the substances utilized for the rise of metabolism caused by the vitamin itself.

That adrenaline decreases the utilization of blood sugar in the peripheral tissues has been indicated by Cori & Cori [1928], by Sahyun & Luck [1929], and by Cori *et al.* [1930].

An alternative explanation for the parallelism between the rate of glycogen mobilization and the degree of saturation of the body with vitamin C under the influence of adrenaline is the possibility that the vitamin in the reduced form protects the adrenaline from rapid oxidation and so, by increasing the saturation of the body with the former, a certain dose of the latter will be rendered more effective in producing the glycogenolysis. Such a relationship between the two substances in the case of the suprarenals has been suggested by different investigators owing to the co-existence of the two substances in the same organ and to the reduction in the amount of both stated by some to occur in scurvy. According to Deutsch & Schlapp [1935], however, there does not appear to be any close relationship between the two substances. Further, the finding of Johnson & Zilva [1934] that the oxidized form of ascorbic acid is not excreted in the urine even after the ingestion of ascorbic acid in that form may at present stand against the latter explanation.

Considering, as a whole, the results obtained when trying the effects of starvation for 24 hours, of insulin and starvation, of insulin and feeding and of adrenaline and starvation, it is found that the apparent variations in these four cases can be explained on the assumption that the vitamin is utilizable in some way during the metabolism of the different substances, so that there is more utilization whenever there is rise in metabolism and less utilization when the metabolism is relatively lowered. Thus, in the case of starvation for 24 hours when the metabolism is relatively lower than when the animals are fed the utilization of the vitamin becomes less and the amount saved causes a rise in the vitamin excretion in the urine. After adrenaline administration, a rise in metabolism was indicated by Sandiford [1920] and by Cori & Cori [1928] and at the same time there is no evidence of increased synthesis of the vitamin; therefore, the increased utilization of the latter accompanying the rise in metabolism results in a remarkable fall in the amount excreted in the urine. In the two cases of insulin and starvation for 24 hours and insulin and feeding the metabolism is raised but there is more continual rise in the second case than in the first owing to the continual supply of metabolites; at the same time there is evidence of increased synthesis of the vitamin under the influence of insulin, consequently the excess in the utilization of the vitamin is supplied by its synthesis. In the first case the synthesis exceeds the utilization resulting, therefore, in a rise of the vitamin in the liver, whilst in the second case the vitamin synthesized in the liver under the influence of insulin does not accumulate in that organ but is transferred to the tissues to meet the requirements of the increased metabolism. In spite of the fact that there is a rise in metabolism, the level of excretion of the vitamin in the urine is high in both cases, which is contrary to the case of adrenaline, and may thus indicate a continually raised charge of the blood by the synthesized vitamin.

SUMMARY.

1. The total vitamin C content of the suprarenals and of the livers of adult rats of initial body weight 220–230 g. is constant whether the animals are provided with an ordinary or with a scorbutic diet.

On a scorbutic diet, rat's suprarenals show an increase in size and in weight which is noticeable only for a certain period.

2. When the ordinary diet is replaced by a scorbutic one, the level of excretion of vitamin C in the urine of rats rapidly falls and remains low for a certain

period, after which it gradually increases owing to the enhancement of the synthetic process, but remains at a lower level than on the ordinary diet.

3. Starvation for 24 hours does not alter the vitamin C content of the suprarenals or of the livers of rats previously kept on a scorbutic diet, but it causes an increase in the amount excreted in the urine.

4. Injection of insulin into rats previously kept on a scorbutic diet increases the vitamin content of the liver, probably owing to increased synthesis. The content of the suprarenals is not affected.

5. Adrenaline does not alter the vitamin C content of the suprarenals or of the liver of rats previously kept on a scorbutic diet, but it lowers the level of excretion in the urine.

6. The rate of glycogenolysis caused by adrenaline runs parallel with the degree of saturation of the body with vitamin C. It is suggested that this degree of saturation is a direct factor in determining the rate of glycogenolysis, or that the vitamin in the reduced form protects the adrenaline from rapid oxidation.

7. The vitamin synthesis by rats kept on a scorbutic diet may be sufficient to insure freedom from scurvy while producing a relatively lower saturation of the body than when the animals are kept on a diet containing the vitamin.

8. It is suggested that vitamin C is utilized in some way during the metabolism of the different substances, so that there is a parallelism between the extents of the two processes.

We wish to express our thanks to Prof. G. V. Anrep and Dr A. Hassan for the encouragement and the interest they have shown in this work.

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CLXXXV. METABOLISM OF SULPHUR.

II. THE QUANTITATIVE DETERMINATION OF CYSTINE IN NORMAL URINE.

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In the first paper of this series [Medes & Padis, 1936] a method was described for the quantitative determination of cystine in solutions, the basis of which was the precipitation of the cystine by cuprous chloride, its recovery as cysteine from the copper mercaptide by the use of hydrogen sulphide and its quantitative determination with phospho-18-tungstic acid. The present paper concerns itself with the application of this method to the estimation of cystine in normal urine.

That the compound so determined consists at least in part of cystine has been demonstrated by the isolation of cystine from normal urine by cuprous chloride precipitation.

Isolation of cystine from normal urine. All reagents were prepared as in the method for quantitative determination of cystine reported in the previous paper. Freshly voided urine was brought to pH about 4·6 with acetic acid and preserved in a refrigerator at about 5° until used (not longer than 6 hours). The urine was filtered to remove any uric acid and inorganic salts that had precipitated. To 3 litres of urine was added an equal volume of the sodium acetate-acetic acid buffer at pH 4·6, followed by 25 g. of cuprous chloride dissolved in the KCl-HCl mixture, added in a slow stream with vigorous stirring. The precipitate was allowed to settle for several hours or overnight and the supernatant liquid siphoned off. At this stage the remaining solution including the precipitate was transferred to a small cylinder to permit further settling of the precipitate. After 2-3 hours as much as possible of the supernatant liquid was decanted, the syrupy precipitate was transferred to 100-ml. centrifuge-tubes (usually 3) and centrifuged at first at slow speed, then more rapidly for about ½ hour. The preliminary slow centrifuging causes a layering of the precipitate, the lower whitish layer consisting chiefly of the excess cuprous chloride, and the upper greyish layer containing the copper mercaptide. The latter was scraped away, transferred to a 1-l. beaker and stirred into about 700 ml. of the buffer solution, and 5 g. of cuprous chloride dissolved in the KCl-HCl solution were again added. The precipitate was allowed to settle, the supernatant liquid decanted and the precipitate centrifuged down as before.

The precipitate was stirred into 200 ml. of 0·1 N H_2SO_4 and decomposed with H_2S. The copper sulphide was removed by filtration with suction, the filtrate was decolorized with norite and the H_2SO_4 removed with $BaCl_2$, the process being interrupted at pH about 6, thus assuring a slight excess of H_2SO_4. The clear almost colourless filtrate was concentrated under reduced pressure to a volume of about 10 ml., cooled and filtered. The precipitate was discarded and the filtrate, after being brought to pH about 6·5 with $NaHCO_3$, further concentrated by standing in a warm room or in a desiccator. Crystals of cystine sometimes appeared after several days, sometimes only after a number of redilutions and reconcentrations. The crystals assumed various forms, usually needles or minute prisms. These were filtered off, dissolved in hot dilute HCl, the solution neutralized with $NaHCO_3$ and again concentrated. This procedure was repeated several times before the hexagonal crystals characteristic of pure cystine could be observed. These hexagonal crystals did not appear suddenly but were preceded by a transitional stage in which the spherules of minute prisms underwent a leaf-like lobulation, each leaf gradually approaching the hexagonal form until the mass was transformed into a spherule of plates showing striations corresponding to the original crystalline structure. After several recrystallizations the clear, hexagonal crystals appeared.

This procedure was repeated twelve times before sufficient crystals were obtained for identification. After further purification they gave, following reduction, the typical reactions of cysteine—the Sullivan reaction, the nitroprusside test and a positive test with phospho-18-tungstic acid. Optical rotation, 1 g. in 100 ml. solution (1 *M* HCl), $[\alpha]^{25^\circ}_{\mathrm{Hg}} = -248^\circ$.

Quantitative determination of cystine. For the quantitative determination of cystine, the method as described in the previous paper was employed, using 8 ml. of urine. To the same urine was added standard cystine solution in varying amounts, within the range found in normal urines. The results are given in Table I expressed in mols per hourly excretion, together with the percentages of added cystine recovered. For comparison determinations were made directly upon the urine by Shinohara's modification [1935] of the Folin-Marenzi method and the results are also given in the table. Six samples from the same individual on a constant diet are represented, the various samples being collected over the period from 7 p.m. to 7 a.m. on 6 consecutive days. The output of cystine determined by the cuprous chloride method averaged $6{\cdot}36 \times 10^{-6}$ *M* (1·53 mg.) per hour, and by Shinohara's method $6{\cdot}49 \times 10^{-6}$ *M* (1·56 mg.) per hour. The individual values by the former ranged from 5·50 to 7·13, and by the latter from 5·37 to 9·36 $M \times 10^{-6}$. By the cuprous chloride method, the recovery of added cystine averaged 89·4 % and by Shinohara's method, 56·5 %.

Table I. *The cystine content of normal urine by the cuprous chloride precipitation method* (*a*) *and by Shinohara's method* (*b*), *together with percentages of added cystine recovered.*

Sample	Vol. ml.	Hrs.	Cystine per hr. $M \times 10^6$	No. of determinations	Cystine added 0·005 *M* ml.	Cystine added 0·001 *M* ml.	% recovered	No. of determinations
1	450	12	*a* 7·13	3	—	—	—	-
			b 9·36	1	—	—	—	—
2	680	12	*a* 6·91	2	1	—	89	4
			b 5·95	1	—	—	—	—
3	450	12	*a* 6·79	2	1	—	88	2
			b 5·37	1	—	—	—	—
4	570	12	*a* 6·82	2	—	0·5	88	2
			a 6·82	2	—	1·0	89	2
			a 6·82	2	—	2·0	88	2
			b 6·80	1	—	1·0	41	2
5	820	12	*a* 5·50	2	—	0·5	86	2
			a 5·50	2	—	1·0	93	2
			a 5·50	2	—	2·0	92	2
			b 5·37	1	—	1·0	66	2
6	735	12	*a* 6·21	2	-	0·5	92	1
			a 6·21	—	—	1·0	92	1
			a 6·21	—	—	2·0	91	1
			b 6·10	2	—	0·5	62	1
			b 6·10	2	—	1·0	65	1
			b 6·10	2	—	2·0	64	1
		Av.	*a* 6·36				89·4	15
		Av.	*b* 6·49				56·5	7

In Table II are recorded the results of a series of tests on 12 samples of urine with results expressed in mg. per 100 ml. and in mg. per hour.

(*a*) *Cuprous chloride precipitation* [Medes & Padis, 1936]. Ascorbic acid is not precipitated by cuprous chloride under these conditions. Glutathione (Hoffman La Roche) was subjected to the same process and failed to precipitate. Ergothioneine and uric acid, although precipitated by cuprous chloride, do not

Table II. *Determination of S-S compounds in urine by various methods.*

	Medes		Shinohara		Virtue & Lewis		Medes-Sullivan		Sullivan*		Rossouw & Wilken-Jorden	
Subject	mg. per 100 ml.	mg. per hr.	mg. per 100 ml.	mg. per hr.	mg. per 100 ml.	mg. per hr.	mg. per 100 ml.	mg. per hr.	mg. per 100 ml.	mg. per hr.	mg. per 100 ml.	mg. per hr.
a	4·20	0·87	7·82	1·62	10·10	2·10		—		—	—	—
a	0·84	0·92	2·88	3·05	1·54	1·63		—	—	—	—	—
a	1·56	0·92	4·08	1·67	3·84	2·26		—	—		—	—
a	2·96	0·92	3·80	1·17	6·59	2·03	—		—		—	—
a	1·07	1·28	3·24	4·12	1·54	1·84		—		—	—	—
a	1·90	0·95	0·96	0·48	2·56	1·28	—	—	5·44	2·72	1·50	0·75
a	1·44	1·03	2·76	1·93	2·56	1·79	1·34	0·94	4·00	2·80	1·29	0·90
a	4·68	1·08	4·82	1·09	7·04	1·62	3·65	0·84	9·91	2·28	3·13	0·72
a	2·06	1·08	2·57	1·34	3·52	1·84	—	—	6·03	3·15	1·62	0·85
b	1·80	1·37	1·97	1·50	2·11	1·60	1·67	1·17	4·45	3·38	1·70	1·29
b	1·10	1·21	2·57	2·83	1·47	1·62	—	—	4·35	4·79	1·00	1·10
b	1·66	1·27	1·97	1·50	2·50	1·91	—	—	4·65	3·55	1·35	1·03
Av.		1·08 ±0·14		1·86 ±0·75		1·71 ±0·22		0·98 ±0·12		3·24 ±0·57		0·95 ±0·16

* Procedure reported at the Kansas City Meeting of the American Chemical Society and given to the author by private communication.

produce colour with uric acid reagent in acid medium. The range of values determined for the two individuals represented in the table extend from 0·87 to 1·28 mg. per hour in one case and from 1·21 to 1·37 mg. per hour in the other.

(*b*) *Method of Shinohara* [Shinohara, 1935]. All SH and SS compounds of urine except ergothioneine probably respond to this test. Glutathione, if present, would be incompletely determined [Shinohara & Padis, 1936, 1]. The final value is obtained by difference between total intensity of colour produced with phospho-18-tungstic acid in urine and intensity of colour produced with phosphotungstic acid by extraneous reducers alone. In cystinuric urines, the colour developed by extraneous reducers constitutes only a small portion of the total colour, whereas in normal urines that produced by SH *plus* SS becomes the minor factor, and hence a method based on this difference is open to wide possibilities of error. In their own studies on the cystine and cysteine contents of urine, Shinohara & Padis [1936, 2] added amounts of these compounds several times greater than those occurring in normal urines. According to Table II, the hourly output of individual *a* ranged from 0·48 to 4·12 mg. and of the second subject, *b*, from 1·50 to 2·83 mg. In the case of the low value, 0·48, the addition of $HgCl_2$ to the blank produced a heavy cloud which could not be centrifuged out, and the colorimetric reading could only be approximate. In two instances the values are greater than determined for total S-S by the method of Virtue & Lewis. This fact, together with the general irregularity of the results indicates that the Shinohara method is of questionable applicability to normal urines.

(*c*) *Method of Virtue & Lewis* [1934]. According to the authors, this method applies to total S-S, and therefore should give the highest values in the table. The only exceptions are the two instances cited under Shinohara's procedure, and the series by Sullivan's method.

(*d*) *Sullivan's method.* The high values obtained by this method indicate that it is not sensitive enough for direct use in normal urines. The yellow colour of the blank interferes with the pale red of the test.

(*e*) *Method of Rossouw & Wilken-Jorden* [1935]. The values obtained by this method vary from 0·72 to 1·29 mg. per hour in the two individuals and average

81 % of the values obtained by cuprous chloride precipitation with subsequent estimation by the uric acid reagent. 82 % of the cystine added to normal urines was recovered on the average, so that it is uncertain whether the low values represent error of determination or the presence of some other compound, such as homocystine, in normal urine. That pure cystine, as indicated by its optical rotation, was isolated by the cuprous chloride technique, speaks against this possibility.

(*f*) *The Sullivan procedure on the filtrate as recovered by H_2S* [Medes & Padis, 1936]. The values obtained by this method are in fair agreement with those by the procedure of Rossouw & Wilken-Jorden and are open to the same considerations. In both these instances 50–100 ml. of urine must be employed and the mercaptides decomposed in 5 ml. of solution. The technical difficulties of such a procedure add to the uncertainty of interpretation of the results.

Production of colour with phospho-18-tungstic acid by oxidation products of cystine. Since in the process of recovering cysteine from its mercaptide by H_2S small amounts of decomposition products may be formed, it is of importance to ascertain the possible effect of these on phospho-18-tungstic acid. Sulphinic acid and cystine disulphoxide were tested through the courtesy of Dr Toennies and Dr Lavine. Sulphinic acid produces no colour with the uric acid reagent under the conditions of these tests and cystine disulphoxide develops colour slowly, approaching about 25 % of the colour developed by a molar equivalent of cysteine. After the first 20 min. when the colorimetric readings in our experiments are taken, only about half of the final colour is developed. The colour produced by traces of disulphoxide which might be formed would therefore be insufficient to affect significantly the final values.

Cysteine in normal urine. Since the cuprous chloride method does not distinguish between cystine and cysteine, attempts were made by Shinohara's method to ascertain if the latter was present. By Shinohara's method, cysteine is determined by difference between colours developed by freshly voided urine with phospho-18-tungstic acid, and by a sample similarly treated, but containing formaldehyde. All tests were performed within 10 min. after the urine was voided. Of 40 samples, 23 gave positive values for cysteine averaging 3×10^{-7} *M* (0·036 mg.) per 100 ml.; 5 samples showed no difference between the two readings and in 12 instances the difference was negative. It seems doubtful, therefore, if cysteine is present in significant amounts in normal urines.

SUMMARY.

The cystine content of specimens of normal urine has been determined by the cuprous chloride precipitation method followed by recovery of cystine as cysteine by decomposition of the mercaptides with hydrogen sulphide and subsequent estimation of the cysteine with phospho-18-tungstic acid.

In this method cystine is differentiated from ergothioneine and glutathione. Ascorbic acid and uric acid are also excluded.

Cysteine probably is not present except possibly occasionally in traces.

The excretion of cystine, as determined by this method, averaged $5·66 \times 10^{-6}$ *M* per hour, which was approximately 70 % of the total S-S as determined by the iodimetric titration method of Virtue & Lewis.

Determination of cystine by the procedure of Rossouw & Wilken-Jorden gave values approximately 82 % of those by the test as developed by the authors.

Determination of cystine in a filtrate obtained by cuprous chloride precipitation, recovery with H_2S and estimation with the Sullivan reagent gave values approximately in agreement with those obtained by the Rossouw & Wilken-Jorden procedure.

The slightly lower values obtained by these two modifications of the Sullivan method leave open the possibility that some other S-S compound, such as homocystine may be present in urine in small amounts.

The author is indebted to Mrs Kively Padis for making the determinations by the Shinohara and the Medes & Padis methods.

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CLXXXVI. AMYLASE DURING THE GROWTH AND RIPENING OF GRAINS.

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Research conducted by us on the amylase of forming and ripening rye grain [Chrząszcz & Janicki, 1936, 2] has shown that in the forming grain immediately after blossoming amylase exists with all its functions intact, i.e. not only with the saccharifying but also with the liquefying and dextrinizing functions. Hence it does not differ from malt amylase. As the grain develops and ripens, the liquefying and dextrinizing functions dwindle and finally disappear: saccharifying power remains, but lessened. This drop in amylase content or in the power of its various functions could, up to a certain stage of ripening of the grain, be counteracted by means of an eleuto-substance, in the shape of peptone nullifying the inactivating influence of the sisto-substance [Chrząszcz & Janicki, 1933].

Our experiments on the relation of increase in the quantity of active amylase in cereals to the decomposition of protein by the action of added proteases furnished evidence against the views of Ford & Guthrie [1908] and of a number of other workers who stated that corn amylase is bound to protein, and that its liberation and activation can be effected only by means of the decomposition of the protein by the action of proteases. Experiments on the influence of hydrogen sulphide on increase of active amylase in cereals have also definitely shown that these views were mistaken [Chrząszcz & Janicki, 1936, 1].

Since the observation that amylase with its three functions appears in rye grain immediately after blossoming conclusively contradicts the view that whole amylase only appears later, during germination, it was considered of interest to investigate whether this phenomenon likewise appears in other cereals, viz. oats, wheat and barley.

Experimental.

Rye grain was taken immediately after formation and at certain intervals during the process of ripening. The procedure followed was the same as in the research on rye [Chrząszcz & Janicki, 1936, 2]. The grain was ground in a mortar, covered with water to form a 10% extract, which after 20 min. was centrifuged. Amylolytic power was then determined. Peptone was added and extracts prepared; in the case of barley, papain was used as the eleuto-substance and, in that of wheat, NaCl also. The tables give the percentage content of water in the cereals and the percentage of each extract used.

Amylolytic power was examined as in the previous research: thus, the saccharifying power was expressed in ml. $N/20$ iodine, computed for 1 g. of dry grain; the dextrinizing power was given without conversion, and the liquefying power was determined by the method of Lintner & Sollied, and expressed as g. of starch liquefied by 1 g. of dry corn.

Barley.

The outcome of the experiment indicates that during the period of ripening, barley grain has not only saccharifying but also dextrinizing and liquefying powers, although in a much less degree than ripening rye grain. It is also striking

Table I. *Amylolytic power of ripening barley grain.*

				Saccharifying power		Dextrinizing power. Min. to change colour of starch with iodine to			Liquefying power	
Days after blossoming	H_2O content of grain %	Extract %	Peptone added %	ml. N/20 iodine per 1 g. of grain	Change %	Violet-blue	Brownish red	Achro. stage	g. of starch liquefied by 1 g. of grain	Change %
10	63·8	3·62	—	150	—	100	405	Not obtained after 840 min.	6·9	—
			1	150	0·0	100	405	,,	8·1	+17·4
			2	143	− 4·6	100	345	,,	8·1	+17·4
			4	150	0·0	90	340	,,	9·2	+33·3
11	62·5	3·75	—	190	+26·6	100	425	,,	7·0	+ 1·5
			1	192	+28·1	90	425	,,	7·3	+ 5·8
			2	178·1	+18·7	90	425	,,	7·3	+ 5·8
			4	169·1	+12·7	90	425	,,	7·3	+ 5·8
			8	169·1	+12·7	85	365	,,	7·3	+ 5·8
14	41·7	5·83	—	165·3	+ 7·5	135	Not obtained after 840 min.	,,	0·0	−100·0
			2	175·7	+17·1	135	,,	,,	0·0	−100·0
			4	165·3	+ 7·5	135	,,	,,	0·0	−100·0
			8	143·8	− 4·1	140	,,	,,	0·0	−100·0

that the dextrinizing and liquefying powers diminish and disappear in barley much more quickly than in rye, the loss in barley taking place during the "milk" stage.

The following experiment was carried out in order to ascertain whether papain helps to increase the amylolytic power of an extract of barley grain taken 14 days after blossoming.

Table II. *Influence of papain on the amylolytic power of ripening barley grain.*

				Saccharifying power		Dextrinizing power. Min. to change colour of starch with iodine to			Liquefying power	
Days after blossoming	H_2O content of grain %	Extract %	Papain added g.	ml. N/20 iodine per 1 g. of grain	Change %	Violet blue	Brownish red	Achro. stage	g. of starch liquefied by 1 g. of grain	Change %
14	42·0	5·8	—	165	—	135	Not obtained after 840 min.		0·0	—
			1	181	+9·7	135	,,		0·0	
			2	165	0·0	135	,,		0·0	—

Table II shows that papain had no effect on the dextrinizing and liquefying powers of barley grain in the phase of initial lactic maturity: the outcome was the same as with the action of proteolytic enzymes in general on ripe barley grain.

Wheat.

Experiments were then made on wheat grains (Table III).

It follows from Table III that wheat behaves similarly to rye, i.e. it is only in the later phases of ripening that the liquefying power dwindles and disappears. The dextrinizing power, however, remains, but only in very moderate strength.

In order to ascertain whether the dextrinizing powers of other types of wheat are greater or not, an experiment was made with two other varieties, the eleuto-substance used being, in addition to peptone, sodium chloride (Table IV).

Table IV shows that wheat No. 3 has high liquefying and dextrinizing powers accompanied by a considerable content of sisto-substances, whose action can be excluded by using peptone and sodium chloride as eleuto-substances. The last-named acts best when in concentrations up to 1 %; in concentrations of 2 % or

Table III. *Amylolytic power of ripening grains of wheat No. 1.*

Days after blossoming	H_2O content of grain %	Extract %	Peptone added %	Saccharifying power		Dextrinizing power. Min. to change colour of starch with iodine to			Liquefying power	
				ml. *N*/20 iodine per 1 g. of grain	Change %	Violet-blue	Brownish red	Achro. stage	g. of starch liquefied by 1 g. of grain	Change %
10	40·7	5·93	—	227·6	—	30	90	Not obtained after 840 min.	8·0	—
			1	221·0	− 2·9	28	90	,,	11·2	+ 40·0
			2	223·9	− 1·6	28	85	,,	11·2	+ 40·0
			4	225·4	− 0·9	28	85	,,	11·2	+ 40·0
12	46·4	5·36	—	262·1	+15·2	30	100	,,	7·8	− 2·5
			1	259·1	+13·9	28	100	,,	10·9	+ 36·2
			2	256·0	+12·5	28	95	,,	10·9	+ 36·2
			4	231·6	+ 1·8	28	90	800	10·9	+ 36·2
16	38·9	6·11	—	203·1	−12·0	60	265	Not obtained after 840 min.	5·4	− 32·5
			2	210·2	− 7·5	50	180	,,	6·0	− 25·0
			4	203·1	−12·0	50	170	,,	6·0	− 25·0
			6	200·0	−12·1	50	165	,,	6·0	− 25·0
23	35·5	6·45	—	192·1	−15·4	60	400	,,	0·0	−100·0
			2	199·2	−12·4	50	205	,,	4·4	− 45·0
			4	199·2	−12·4	52	175	,,	4·4	− 45·0
			6	183·6	−19·3	55	205	,,	4·4	− 45·0

Table IV. *Influence of peptone and NaCl on ripening wheats Nos. 2 and 3.*

Days after blossoming	H_2O content of grain %	Extract %	% added		Saccharifying power		Dextrinizing power. Min. to change colour of starch with iodine to			Liquefying power	
			Peptone	NaCl	ml. *N*/20 iodine per 1 g. of grain	Change %	Violet-blue	Brownish red	Achro. stage	g. of starch liquefied by 1 g. of grain	Change %
						Wheat No. 2.					
23	48·0	5·2	—	—	238·6	—	55	140	Not obtained after 840 min.	7·7	—
			2	—	250·0	+4·8	50	130	,,	9·1	+18·2
			4	—	258·7	+8·0	45	120	,,	11·3	+46·7
			—	2	250·0	+4·8	56	175	,,	7·1	− 7·9
			—	4	247·0	+3·9	60	265	,,	5·5	− 28·7
						Wheat No. 3.					
25	38·5	6·15	—	—	216·0	—	20	65	400	13·5	—
			2	—	226·2	+1·9	15	50	320	20·3	+50·4
			4	—	220·2	+1·9	15	50	320	23·3	+72·6
			—	0·5	222·0	+2·7	13	50	350	20·3	+50·4
			—	1·0	218·2	+1·0	12	55	370	23·3	+72·6

more, it decreases the power of the dextrinizing and liquefying functions, at least in the case of wheat No. 2. The dextrinizing power of wheat No. 3 was so great that starch was easily dextrinized to the achro-stage with an extract of this wheat.

Oats.

Oats during germination have high dextrinizing and liquefying but relatively low saccharifying powers. It was therefore of interest to examine these three functions in the ripening grain (Table V).

It can be seen from Table V that in ripening oat grain there is a more or less similar relation between the various amylolytic functions to that in malt, viz. relatively high liquefying and dextrinizing and only moderate saccharifying powers. In the case of ripening oat grain, the solutions of dextrinized starch likewise have a greenish tinge just as is often the case with germinating oats (malt of oats).

Table V. *Amylolytic power in ripening oats.*

				Saccharifying power		Dextrinizing power. Min. to change colour of starch with iodine to			Liquefying power	
Days after blossoming	H_2O content of grain %	Extract %	Added peptone %	ml. $N/20$ iodine per 1 g. of grain	Change %	Violet-blue	Brownish-red	Other stages	g. of starch liquefied by 1 g. grain	Change %
9	40·9	5·91	—	142·2	—	34	150	375 (green)	11·3	—
			2	145·2	+ 2·1	34	90	300 (375, blue)	13·0	+15·0
12	40·8	5·92	—	114·4	−24·3	85	220	Achro. not obtained after 840 min.	10·0	−11·5
			3	115·5	−23·1	40	100	365 (green)	10·6	− 6·2
			6	134·5	− 5·4	30	90	840 (achro. not obtained)	10·0	−11·5
14	40·0	6·0	—	121·0	−15·5	63	145	840 (achro. not obtained)	9·2	−18·6
			3	118·6	−16·6	38	100	400 (achro.)	10·4	− 7·9
			6	114·2	−19·7	35	100	840 (achro. not obtained)	11·0	− 2·7
						Oats No. 2.				
19	55·5	4·45	—	140·4	—	43	140	840 (achro. not obtained)	11·8	—
			3	197·0	+40·3	35	80	325 (achro.)	17·3	+46·6
			6	187·2	+33·4	30	80	840 (achro. not obtained)	22·0	+86·5
27	42·0	5·8	—	94·3	−32·9	62	225	840 (achro. not obtained)	5·8	−50·9
			3	103·2	−26·6	68	200	700 (achro.)	6·1	−48·3

DISCUSSION.

The results of these experiments on barley, wheat and oats confirm and greatly extend the research carried out with forming and ripening rye grain. It appears that after the blossoming of the cereal, all three amylolytic functions (liquefying, dextrinizing and saccharifying) appear in the forming grain, that is to say exactly as in germinating barley (in malt). This phenomenon is therefore a general one, appearing in all species of cereal. These three amylolytic functions are not maintained, however, at a constant level, but on the contrary, as the grain develops and ripens, the various functions gradually decrease in activity (dextrinizing and liquefying powers particularly, as these almost completely disappear in the later phases of ripening). This decrease in the strength of the various functions is a reversible feature up to a given phase of development of the grain since the primary activity can be restored with the help of eleuto-substances (peptone or weak solutions of salt). Evidently sisto-substances accumulate in the ripening grain and act with steadily increasing effect. Their activity can be counteracted by the influence of the eleuto-substances mentioned, but only up to a certain stage of development of the grain and in a great measure individually in dependence on the type of cereal and its variety. Evidently at a certain stage of development these sisto-substances collect in such large quantity, or their ability to bind amylase increases to such an extent, that their activity cannot be nullified by the eleuto-substances; this in turn evokes an almost complete loss of dextrinizing and liquefying powers.

It is of interest that in forming barley grain the loss of these two functions takes place during the "milk" stage, but in other cereals this happens much more slowly and the dextrinizing and liquefying functions finally disappear only when the grain is almost fully ripe. This phenomenon is in agreement with our former observations, viz. that barley and buckwheat have the highest content of sisto-substance, evidently produced in great quantity concurrently with the beginning of the grain formation.

These experiments confirm the view expressed when investigating rye, viz. that Nordh & Ohlsson's [1932] hypothesis, affirming that the so-called α-amylase forms only during the germination of grains, is erroneous.

Purr's [1934] view that the disappearance of dextrinizing power of amylase is due to the oxidation of ascorbic acid during ripening, likewise appears to be untenable. The experiments on ripening grains, those on rye and those on wheat, oats and barley, fail to confirm this author's suppositions. The gradual diminution of the dextrinizing function can be counteracted with peptone and even with NaCl acting as eleuto-substances. This fact points to the adsorptive inactivation of this function, as it is difficult to suppose that by such means it is possible to reverse the process of oxidation of ascorbic acid during the preparation of the grain extract. Further research will elucidate whether the disappearance or decrease of the dextrinizing and liquefying functions is evoked exclusively by the adsorption-elution of amylase, so often indicated by us, or whether apart from this, still other factors enter into consideration.

SUMMARY.

1. In wheats, oats and barley, as is the case with rye, whole amylase is found with its three functions intact (liquefying, dextrinizing and saccharifying) immediately after blossoming from the moment the grain is formed.

2. As the grain ripens, the liquefying and dextrinizing functions gradually disappear, and the saccharifying function undergoes partial decrease.

3. This disappearance of the liquefying and dextrinizing functions of amylase is above all of an adsorptive nature and can be counteracted by eleuto-substances (peptone or NaCl). The latter give better results with wheat at concentrations of 0·5–1·0 %. The disappearance of dextrinizing and liquefying functions could not be counteracted by the action of papain.

4. The activities of the various functions of amylase and the speed of their diminution and disappearance, as also their liberation by the action of eleuto-substances, are an individual trait of cereal and its various species. With barley, which possesses the highest quantity of sisto-substances, the diminution of the various functions of amylase commences earliest, i.e. during the early stages of ripening.

5. Nordh and Ohlsson's view that α-amylase is formed only during the germination of grains, as also Purr's statement that the gradual total loss of dextrinizing power of amylase is due to the oxidation of ascorbic acid, were not confirmed by our previous experiments on rye or by the present ones on the development of amylase in ripening barley, wheat and oats.

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CLXXXVII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

L. RAVENELIN (3-METHYL-1:4:8-TRIHYDROXY-XANTHONE), A NEW METABOLIC PRODUCT OF *HELMINTHOSPORIUM RAVENELII* CURTIS AND OF *H. TURCICUM* PASSERINI.

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IN a recent series of communications from these laboratories we have recorded the isolation from the dried mycelium of several different species of *Helminthosporium*, when grown on a synthetic medium containing glucose as the sole source of carbon, of a number of hitherto unrecorded polyhydroxyanthraquinones [Charles *et al.* 1933; Raistrick *et al.* 1933; 1934]. These mould metabolic products included helminthosporin (2-methyl-4:5:8-trihydroxyanthraquinone) from *H. gramineum* Rabenhorst and *H. catenarium* Drechsler; catenarin ($\alpha\beta$-(hydroxymethyl)-1:5:8-trihydroxyanthraquinone) from *H. catenarium* Drechsler, *H. gramineum* Rabenhorst and *H. velutinum* Link; cynodontin (2-methyl-1:4:5:8-tetrahydroxyanthraquinone) from *H. cynodontis* Marignoni, *H. euchlaenae* Zimmermann, and *H. avenae* Eidam, and tritisporin (6(or 7)-(hydroxymethyl)-1:3:5:8-tetrahydroxyanthraquinone from *H. tritici-vulgaris* Nisikado. In some cases these polyhydroxyanthraquinones are present in surprisingly large amounts, comprising in the case of *H. gramineum* about 30 % of the weight of the dried mycelium.

During the investigation of some 40 species of *Helminthosporium* it was found that, in the cases of *H. Ravenelii* Curtis and *H. turcicum* Passerini, although solvent extraction of their dried mycelia yielded considerable amounts of a yellow crystalline material, this was not a polyhydroxyanthraquinone. This substance, for which the name *ravenelin* is proposed, has now been purified and characterized as 3-methyl-1:4:8-trihydroxyxanthone, thus affording the first recorded instance of the production by any of the lower fungi of a polyhydroxyxanthone. Two naturally occurring hydroxyxanthones have been previously examined, namely (*a*) euxanthone (1:7-dihydroxyxanthone), which occurs as the glucoside mangiferin in the leaves of *Mangifera indica* L., the Indian mango tree, and, coupled with glucuronic acid, as euxanthic acid in the pigment "piuri", or Indian yellow, prepared from the urine of cows fed on mango leaves, and (*b*) gentisin (the 3-methyl ether of 1:3:7-trihydroxyxanthone) which is present probably as a glucoside in the rhizomes of *Gentiana lutea* L.

Nisikado [1929] divided the genus *Helminthosporium* into two subgenera on morphological grounds, i.e. the subgenus *Eu-Helminthosporium* and the subgenus *Cylindro-Helminthosporium*. It is perhaps significant that of the 40 species examined biochemically by us which are common to the 24 species examined morphologically by Nisikado, all those species (with one possible exception) which we have found to produce polyhydroxyanthraquinones are included by Nisikado in the subgenus *Cylindro-Helminthosporium*, whilst those species which fail to produce polyhydroxyanthraquinones, including *H. Ravenelii* and *H. turcicum* which produce a polyhydroxyxanthone, are included in the subgenus *Eu-Helminthosporium*.

H. Ravenelii Curtis (syn. *H. hoffmanni* Berkeley and Curtis, *H. tonkinense* Karsten & Roumeguére, and *H. crustaceum* Hennings), although of very little economic importance, has become one of the best known members of the genus *Helminthosporium* because of its conspicuousness and wide occurrence in many of the warmer regions of the world. It was first described in 1848 by Curtis who noted its abundant distribution in North and South Carolina as a parasite on the grass *Sporobolus indicus* R. Since that time it has been reported by many different mycologists, on *Sporobolus indicus* growing in different parts of North and South America and China. A full account of it is given by Drechsler [1923].

H. turcicum Passerini (syn. *H. inconspicuum* Cooke & Ellis) is the cause of a disease of maize (*Zea Mays* L.) commonly known as "leaf blight" and less frequently as "white blast". It appears to have been first noticed in 1876 in Italy by Passerini. Leaf blight disease of maize is of almost universal occurrence, having been reported throughout the maize belt of the United States, in New South Wales, Japan, India, South Africa and Russia. *H. turcicum* has also been reported on cultivated sorghum by Butler [1918] in India, Egypt and China and by other authors in different parts of the world. A full account of it is given by Drechsler [1923].

Chemistry of ravenelin. The intensely yellow crystalline substance, M.P. 267–268° (corr.), was found to be homogeneous; it is optically inactive and has pseudo-acidic characters. The composition, $C_{14}H_{10}O_5$, was established by elementary analysis, mol. wt. determinations (cryoscopic in camphor) and by the preparation and analysis of derivatives such as the triacetyl-, tribenzoyl- and trianisoyl-ravenelins and ravenelin dimethyl and trimethyl ethers.

These experiments also demonstrated the absence of the carboxyl group confirmed by the failure to effect esterification with the help of the usual acid catalysts.

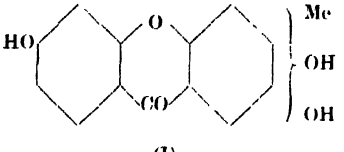
It is difficult to formulate a trihydroxy-compound, $C_{14}H_{10}O_5$, as a derivative of naphthalene, anthracene or phenanthrene, and yet a polycyclic system was clearly indicated. Hence, especially as the function of two oxygen atoms remained undecided and lactonic character was not evinced, we suspected a xanthone structure. A substance of the composition $C_{14}H_{10}O_5$ might be a trihydroxymethylxanthone. Direct evidence of the presence of a methyl group was obtained by means of the Kuhn-Roth micro-method.

According to the conditions of the experiment, ravenelin affords a dimethyl or a trimethyl ether when it is treated with diazomethane in alcoholic-ethereal solution; the dimethyl ether can be further methylated by means of methyl sulphate and sodium hydroxide in aqueous acetone solution.

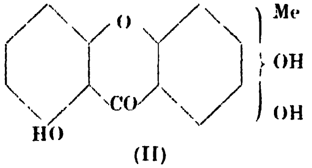
The trimethyl ether is inert towards alkalis and acetic anhydride and its most interesting property is its basic character. It is a rather strong oxonium base and a *ferrichloride*, $C_{17}H_{17}O_5.FeCl_4$, was isolated and analysed.

This observation gave very strong support to the view that ravenelin is a trihydroxymethylxanthone. Confirmation was obtained by the isolation of the ethyl ether of a substituted phenylxanthydrol after reaction between *O*-trimethylravenelin and phenyl magnesium bromide.

Degradation of ravenelin proved to be a very difficult operation, but resorcinol was obtained by fusion with potassium hydroxide. The structure could therefore be expressed as (I) or (II).

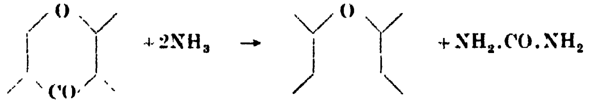


(I)

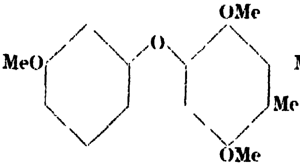


(II)

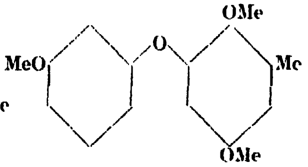
Eventually it was found that the pyrone ring could be opened as the result of submission of ravenelin trimethyl ether to the action of sodamide in boiling xylene solution. The effect was to remove a carbon and an oxygen atom and to introduce two hydrogen atoms. It was evident that the reaction had gone beyond the usual first stage of the Haller process (scission of —CO—R affording —$CONH_2$ and HR) and that, in all probability, the fission of a xanthone ring occurred in accordance with the scheme:



The presumed substituted diphenyl ether was a homogeneous oil, but fortunately it afforded a characteristic crystalline *tetrabromo-derivative.* It will be noticed that structures of the types I and II are derived from one and the same diphenyl ether and as the number of plausible formulae was limited we turned to synthesis in order to decide between them. In this we confined ourselves to substances in which the hydroxyl groups in the trihydroxytoluene nucleus were in *o*- or *p*-relation because ravenelin in alkaline solution is readily oxidized by atmospheric oxygen. The isomeric ethers III, IV and V were prepared by appropriate modifications of Ullmann's method from *m*-methoxyphenol and the requisite dimethoxybromotoluenes.



(III)



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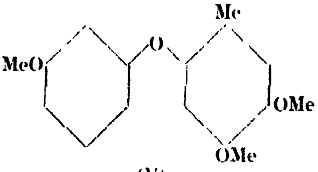
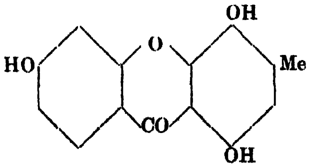
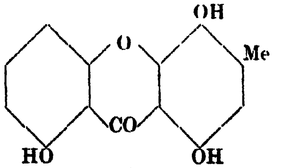


(V)

The isomerides III and V proved to be crystallizable but IV was liquid and identical with the substance obtained from ravenelin trimethyl ether by interaction with sodamide. This important conclusion followed a careful comparison of the tetrabromo-derivatives. It follows that ravenelin is VI or VII.



(VI)

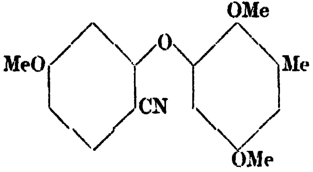


(VII)

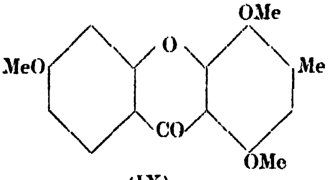
The analytical evidence at our disposal did not distinguish between these possibilities although the formation of a dimethyl ether might be held to support VI because VII contains two hydroxyls in the *o*-position to the carbonyl. However, it is probable that the inactivation of hydroxyl by neighbouring carbonyl is due to chelation and theoretically this implies that one carbonyl group can inhibit the methylation of only one hydroxyl group at a time.

Actually VII must be the correct formula because we have been able to synthesize the trimethyl ether of VI and this substance, although very similar to, is different from *O*-trimethylravenelin.

The condensation of 3-bromo-2:5-dimethoxytoluene with 2-hydroxy-4-methoxybenzonitrile in the presence of potassium hydroxide or ethoxide and copper bronze at 200° affords 2:5-dimethoxytoluene, 2:4-dimethoxybenzonitrile (!) and *2-cyano-3′-methyl-5:2′:5′-trimethoxydiphenyl ether* (VIII) which is hydrolysed by boiling aqueous barium hydroxide to the corresponding *acid.* Hot phosphoryl chloride then effects the ring-closure to *3-methyl-1:4:6-trimethoxyxanthone* (IX).

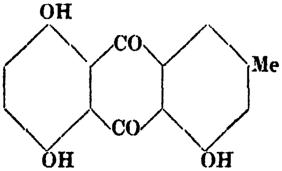


(VIII)

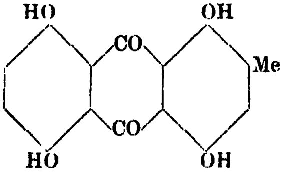


(IX)

It is of interest to compare the constitution of ravenelin (VII) with that of helminthosporin (X) because the methyl group and two of the hydroxyl groups are identically situated in these two metabolic products of species of *Helminthosporium.*



(X)



(XI)

In cynodontin (XI), a congener of helminthosporin, the fourth hydroxyl corresponds to the third of ravenelin. It is not known whether cynodontin is an oxidation product of helminthosporin or whether the latter is derived from the former by reduction, but a Dakin-type oxidation of XI followed by reduction could yield VII. This particular hypothesis is naturally a pure speculation and is mentioned only as an example of the kind of relation that may exist between these natural products.

Experimental.

Cultures. The culture of *Helminthosporium Ravenelii* Curtis (L.S.H.T.M. Cat. No. Ag. 138) used throughout this work was purchased from the Centraalbureau voor Schimmelcultures, Baarn, Holland, in June 1933. It was received by them in November 1929 from Nisikado, who isolated it in Japan from *Sporobolus* sp. (strain No. 216). The culture of *H. turcicum* Passerini (L.S.H.T.M. Cat. No. Ag. 124) used was purchased from Baarn in May 1932. It was received by them in November 1929 from Nisikado, who isolated it in Japan from *Zea Mays* L. (strain No. 164).

A. *Cultural conditions and course of metabolism.*

35 litres of Czapek-Dox medium of the following composition were used: glucose, 50 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·01 g.; distilled water, 1 litre. This was distributed in 350 ml. amounts in 100 one-litre conical flasks and sterilized. After sterilization a measured quantity, previously determined, of sterile *N* NaOH solution was added to each flask to bring the *p*H of the medium to 8·0. Each flask was then sown with the same volume of a suspension in sterile Czapek-Dox solution of a pure culture of *H. Ravenelii* grown on Czapek-Dox agar slopes for 2–3 weeks. The inoculated flasks were incubated at 24° and the mixed contents of 5 flasks were analysed after varying incubation periods. The methods used for treatment of the mycelium are described in section C. The results obtained are given in Table I in which the weights recorded are those obtained from 5 flasks in each case. After 69 days' incubation the remaining 65 flasks were taken off and the figures given in Table I for this incubation period are calculated from the results obtained.

Table I.

Incubation period in days	% glucose in metabolism solution by polarimeter	Wt. of mycelium g.	Wt. of crude ravenelin g.	M.P. of crude ravenelin ° C.	Wt. of crude fat g.
21	4·66	2·42	Trace	Very indefinite	0·21
28	4·09	5·16	0·15	253–6	0·33
35	2·82	11·77	0·24	258–263	1·10
42	2·48	12·41	0·30	255–8	1·40
49	2·10	12·25	0·32	252–5	1·18
61	1·50	10·67	0·37	254–6	1·15
66	0·61	16·58	0·38	255–7	2·33
69	0·64	15·23	0·34	259–261	2·91

B. *Effects of temperature and* p*H on yield of ravenelin.*

A batch of Czapek-Dox medium of the composition given above was made up, distributed in 350 ml. quantities and sterilized. After sterilization some of the flasks were inoculated with *H. Ravenelii* without further treatment (*p*H of medium = 4·2) while some were adjusted to *p*H 6·0 and the remainder to *p*H 8·0 before inoculation. Five flasks at each *p*H were then incubated at 24°, 5 at 27° and 5 at 30°, in all cases for 52 days. The results obtained are summarized in Table II and refer to yields from 5 flasks in all cases.

Table II.

Initial *p*H	Incubation temperature ° C.	Residual glucose by polarimeter %	Wt. of mycelium g.	Wt. of crude fat g.	Wt. of crude ravenelin g.	M.P. of crude ravenelin ° C.
4·2	24	2·21	12·1	1·51	1·72	255–8
6·0	24	1·43	14·1	1·84	1·08	260–1
8·0	24	0·90	15·2	2·50	0·50	261–3
4·2	27	0·90	13·8	1·71	1·71	258–61
6·0	27	1·25	13·4	1·12	1·20	246–50
8·0	27	1·33	14·1	0·90	0·86	246–51
4·2	30	2·47	10·1	0·40	1·28	249–58
6·0	30	2·56	9·0	0·29	0·83	249–56
8·0	30	1·76	11·2	0·25	0·73	252–56

It is clear from these figures that the optimum conditions for the production of ravenelin by *H. Ravenelii* on Czapek-Dox medium are an incubation temperature of 24–27° and an initial pH of 4·2. However, since ravenelin, in quantities sufficient for its chemical investigation, had already been prepared before these findings were available, no use was made of them for the large-scale preparation of ravenelin except for the preparation of one batch (see Table III, batch 6).

C. *Preparation of ravenelin.*

Several batches of ravenelin have been prepared and in each case the medium (adjusted before inoculation to pH 8·0 except batch 6, Table III, in which the initial pH was 4·2), cultural conditions etc. employed were the same as are described in section A. 100 flasks were used in each batch. At the end of the incubation period the flasks were similar in appearance, the mould growth having a crumpled dark grey to buff surface, with a brown or red margin and a khaki-brown reverse, while the metabolism solution was red-brown in colour. The mycelium was filtered from the metabolism solution, squeezed, washed with cold water, pressed and dried in a vacuum oven at about 40°. It was ground to a fine powder in a coffee mill and extracted for 1 working day with light petroleum (B.P. 40–50°) to remove most of the fat and sterols. The light petroleum-extracted mycelium was dried, re-ground and exhaustively extracted under a reflux condenser with boiling chloroform. (Ethyl ether is almost as efficient.) The crude ravenelin separating from the chloroform (or ether) extracts was filtered, washed, dried and weighed. The solvent was removed from the chloroform (or ether) mother-liquors and the residue was extracted with a small amount of light petroleum (40–50°). The undissolved residue consisted, as did the material separating from the original light petroleum extract, mainly of ergosterol together with small amounts of ravenelin. The combined light petroleum extracts yielded, on evaporation, considerable amounts of crude fat.

Further extraction with acetone or methyl alcohol of the chloroform-extracted mycelium yielded small amounts of mannitol.

The experimental details of 6 batches are summarized in Table III in which all figures refer to 100 flasks.

Table III.

Batch	Incubation period in days	% glucose in metabolism solution by polarimeter	Wt. of mycelium g.	Wt. of crude ravenelin g.	Wt. of crude fat g.	Wt. of crude ergosterol g.
1	52	0·90	307	10·0	53·0	1·75
2	53	0·92	307	10·0	49·6	1·31
3	56	0·63	324	11·2	63·8	2·58
4	67	0·90	300	8·9	80·5	2·02
5	67	0·47	295	11·0	69·1	3·97
6	68	0·94	266	24·8	20·4	4·46

The M.P. of the crude ravenelin was in all cases about 260°.

Isolation of ravenelin from H. turcicum. 100 flasks (35 litres) of Czapek-Dox medium (see p. 1307), initial pH 4·2 unadjusted, were sown with *H. turcicum* and incubated at 24° for 60 days; residual glucose by polarimeter, 1·52 %; weight of dried mycelium, 273 g. The ground mycelium was extracted as described in section C for *H. Ravenelii*, giving 10·6 g. of a mixture of crude fat and crude ergosterol and 0·75 g. crude ravenelin, M.P. 258–261°, alone or mixed with ravenelin from *H. Ravenelii.*

Chemistry of ravenelin.

Ravenelin (batch 1 *ex H. Ravenelii*) had M.P. 265–266° (corr.) raised to 267–268° (corr.) by crystallization from acetone-chloroform as microscopic needles and prismatic needles (found: C, 65·3, 65·3, 65·3, 64·8; H, 4·0, 3·8, 3·9, 4·0%;

mol. wt. (cryoscopic in camphor), 258, 255) or pyridine (found: C, 65·5; H, 4·0. $C_{14}H_{10}O_5$ requires C, 65·1; H, 3·9%; mol. wt., 258). Found on sample *ex H. turcicum*: C, 65·3; H, 4·2%. On oxidation with chromic acid (Kuhn-Roth method) evidence of the presence of one side-chain methyl group was obtained (found: 89·9, 77·3% of $1C_2H_4O_2$). The intensely yellow substance gives yellow solutions in organic solvents in most of which it is sparingly soluble. It is freely soluble in cold pyridine and hot acetic acid, moderately readily soluble in hot alcohol and acetone and sparingly soluble in hot benzene and light petroleum.

It is insoluble in boiling aqueous sodium acetate but dissolves in hot aqueous sodium carbonate to a brownish yellow solution. The yellow solution in oxygen-free aqueous NaOH quickly becomes deep brown on shaking with air. The ferric chloride reaction in alcoholic solution is an intense dull greenish brown. On heating the orange-yellow solution in sulphuric acid it becomes redder and deeper in tone and then orange-brown.

Ravenelin itself does not appear to form an oxonium salt in contact with concentrated hydrochloric acid.

O-Triacetylravenelin. A mixture of ravenelin (2 g.), acetic anhydride (20 ml.) and pyridine (0·3 ml.) was heated on the steam-bath for 4 hours. The derivative isolated in the usual manner crystallized from acetic acid in slender, colourless needles, M.P. 204–205° (corr.) (2 g.). (Found: C, 62·5; H, 4·2%. $C_{20}H_{16}O_8$ requires C, 62·4; H, 4·2%.) A determination of acetyl groups by the Kuhn-Roth oxidation process indicated the presence of three acetate residues and the side-chain methyl group. (Found: 90·2, 89·8% of $4C_2H_4O_2$.) The substance was optically inactive in chloroform solution. It is sparingly soluble in cold alcohol and benzene but readily soluble in the hot solvents. The material crystallized from acetic acid dissolved in cold acetone and quickly separated again in the form of hair-fine needles.

O-Tribenzoylravenelin. Ravenelin (0·5 g.) was benzoylated in pyridine solution by means of benzoyl chloride (2·5 ml.), at first in the cold and eventually by boiling the mixture for 2 min. The product crystallized on cooling and was collected, washed and crystallized from pyridine. It separated as colourless, elongated, microscopic, prismatic needles with pointed ends, M.P. 255° (corr.). (Found: C, 73·6; H, 3·9%. $C_{35}H_{22}O_8$ requires C, 73·6; H, 3·9%.) The derivative is sparingly soluble in boiling alcohol, moderately readily soluble in hot acetic acid and readily soluble in warm pyridine.

O-Trianisoylravenelin. This derivative was prepared like the benzoate. It crystallized from pyridine and then from acetic acid as colourless, flat, friable needles, M.P. 216–218° (corr.). (Found: C, 69·3; H, 4·5%. $C_{38}H_{28}O_{11}$ requires C, 69·1; H, 4·3%.) The substance was insoluble in aqueous alkalis, and ferric chloride-negative, so that apparently it contained no free hydroxyl group; it gave consistently low values in methoxyl estimations.

Action of nitric acid on ravenelin. Vigorous oxidation occurred under most conditions but the only products isolated were oxalic acid and a sparingly soluble nitro-derivative obtained in insufficient amount for investigation.

Tribromoravenelin. When ravenelin (0·2 g.) was brominated in hot acetic acid by means of bromine (1 g.) the derivative crystallized on cooling and was recrystallized from acetic acid in the form of long, thin, deep yellow, opaque splinters, not possessing a definite melting-point. (Found: Br, 48·0%. $C_{14}H_7O_5Br_3$ requires Br, 48·4%.)

Fusion of ravenelin with alkalis and other experiments. Only unchanged material was obtained as the result of attempted reduction of ravenelin by means of hydriodic acid and phosphorus at 180° or by distillation over zinc dust in a stream of hydrogen. The attempted oxidation of the triacetate by means of chromic acid in acetic acid solution was also fruitless.

A mixture of ravenelin (3 g.), sodium hydroxide (10 g.), potassium hydroxide (10 g.) and water (20 ml.) was heated in a nickel crucible (aluminium block at 240–275°) for 2 hours and finally for 15 min. at a higher temperature (block at 340°, melt at 250°). The product was dissolved in water, acidified, saturated with ammonium sulphate and extracted with ether. The syrup isolated in this way, which gave a strong fluorescein reaction test for resorcinol, was treated with methyl sulphate and aqueous sodium hydroxide in excess, and the resulting oil isolated by means of ether. It had B.P. 210–211°/736 mm. (corr.) (yield, 0·3 g.). (Found: C, 69·9; H, 7·5 %. Calc. for $C_8H_{10}O_2$: C, 70·0; H, 7·3 %.) The substance was recognized as resorcinol dimethyl ether by its odour and B.P. (authentic specimen, B.P. 209–214°/736 mm.) and by the preparation of the dibromo-derivative, M.P. 142–143° (corr.) alone or mixed with an authentic specimen. The other products of the alkali fusion could not be isolated in a pure condition.

Methylation of ravenelin. Direct processes using methyl sulphate and alkalis gave unsatisfactory results.

(*a*) A suspension of ravenelin (3 g.) in absolute alcohol (200 ml.) was treated with diazomethane (from 10 g. of nitrosomethylurea, method of Arndt & Amende [1930]) for 48 hours at room temperature. The solid products were isolated and treated again in exactly the same way. The solvent was then removed by distillation and the residue extracted with hot alcohol (some insoluble residue). The cooled solution deposited long, yellow, hair-like needles, M.P. 285–287° (corr.) (0·15 g.). (Found: C, 66·7; H, 5·0; MeO, 21·8 %. $C_{16}H_{14}O_5$ requires C, 67·1; H, 4·9; 2MeO, 21·7 %.)

(*b*) The crude dimethyl ether (0·3 g.) was dissolved in a little acetone and methyl sulphate (5 ml.) added. The mixture was then treated alternately with aqueous NaOH and methyl sulphate until the aqueous alkaline solution was no longer intensely yellow in colour. The acetone was then evaporated and the solid collected (0·25 g.) and crystallized from alcohol. It separated as colourless prisms, M.P. 178–179° (corr.).

(*c*) The same substance was obtained by the action of diazomethane on ravenelin in alcohol containing a little water. Ravenelin (2 g.) in 99·5 % alcohol (300 ml.) was treated with diazomethane (from 20 g. of nitrosomethylurea using undried ether). After keeping for 48 hours at the room temperature the excess of diazomethane was decomposed by acetic acid and the solvents removed. The solids obtained on addition of water were collected (1·95 g.) and dissolved in alcohol (0·2 g., residue). On cooling the solution deposited colourless prisms, M.P. 178–179° after recrystallization from alcohol. (Found: C, 68·4, 68·1; H, 5·7, 5·4; MeO, 31·9 %. $C_{17}H_{16}O_5$ requires C, 68·0; H, 5·4; 3MeO, 31·0 %.) The procedure described under (*b*) above was applied to the material in the mother-liquors and in this way 0·9 g. of the trimethyl ether was recovered.

The derivative dissolves in cold conc. HCl to an intense orange solution, the colour being discharged by the addition of water or alcohol.

The hydrobromide crystallized as orange-yellow needles when the substance was dissolved in acetic acid containing hydrogen bromide. This trimethylravenelin was unchanged after treatment with hydroxylamine, semicarbazide, boiling aqueous sodium hydroxide or barium hydroxide or alcoholic potassium hydroxide and acetic anhydride under various conditions.

Ferrichloride. Trimethylravenelin (0·15 g.) was dissolved in conc. HCl and a solution of $FeCl_3$ (0·3 g.) in conc. HCl added. The derivative crystallized from acetic acid containing HCl and $FeCl_3$ as red prisms, M.P. 174–175° (corr.). (Found: C, 40·8; H, 3·8 %. $C_{17}H_{17}O_5FeCl_4$ requires C, 40·8; H, 3·4 %.)

Nitrodimethylravenelin. After the addition of HNO_3 (1·5 ml. sp.gr. 1·42) to a solution of trimethylravenelin (0·1 g.) in acetic acid (5 ml.), the nitro-derivative crystallized (0·1 g.) in a few minutes in the cold. It separated from acetic acid in flat, elongated, rather irregular prisms, M.P. 224–226° (corr.). (Found: C, 58·4; H, 4·0; N, 4·4 %. $C_{16}H_{13}O_7N$ requires C, 58·0; H, 3·9; N, 4·2 %.) The derivative is orange in mass and pale yellow by transmitted light under the microscope. It is insoluble in aqueous NaOH but its alcoholic solution becomes intensely greenish brown on the addition of ferric chloride and this reaction confirms the loss of a methyl group indicated by the analytical results.

Action of sodamide on trimethylravenelin. Very little reaction occurred when the experiment was conducted in boiling benzene solution during 8½ hours. A mixture of trimethylravenelin (2·5 g.), powdered sodamide (10 g.) and xylene (50 ml.) was refluxed (bath at 145–150°) for 7 hours. Ice was added to the cooled mixture and the xylene layer separated, dried and distilled. After the removal of the solvent there remained a nitrogen-free oil (1·5 g.) which distilled at 0·5 mm. (bath at 195°). (Found: C, 70·2; H, 6·6; MeO, 33·9 %. $C_{16}H_{18}O_4$ requires C, 70·0; H, 6·6; 3MeO, 34·0 %.) This *trimethoxymethyldiphenyl ether* could not be induced to crystallize, but it was characterized and identified by means of its tetrabromo-derivative, M.P. 152°, which is described below in connexion with the synthetic specimen of unambiguous constitution.

Ethyl ether of phenyldihydro-O-trimethylravenelin (*ethyl ether of a phenyltrimethoxyxanthydrol*). A solution of trimethylravenelin (0·3 g.) in benzene (10 ml.) was added to one of phenyl magnesium bromide (bromobenzene, 2·4 g.; magnesium, 0·4 g.; ether, 8 ml.) and the mixture was refluxed for 4 hours. The mass was decomposed with water, diluted with benzene and the benzene layer separated and extracted with successive small volumes of conc. HCl until these extracts were pale in colour. The orange-yellow aqueous acid solutions were neutralized with ammonia and the precipitated solid was collected (0·35 g.) and boiled with alcohol. This process ethylated the carbinol and the colourless ethyl ether crystallized in twinned, flat, rhombic plates, M.P. 166° (corr.). (Found: C, 73·6, 73·9; H, 6·4, 6·5 %. $C_{25}H_{26}O_5$ requires C, 73·9; H, 6·5 %.) On treatment with cold aqueous HCl the ether was hydrolysed and an orange-yellow solution of the corresponding xanthylium salt was obtained.

Synthetical experiments bearing on the constitution of ravenelin.

2:5-Dimethoxy-4-bromotoluene. 2:5-Dihydroxy-4-bromotoluene [Clark, 1892] was methylated by means of methyl sulphate and NaOH in aqueous acetone solution. The dimethyl ether crystallized from alcohol in polyhedra, M.P. 90–91° (corr.). (Found: Br, 34·4 %. $C_9H_{11}O_2Br$ requires Br, 34·7 %.)

2:5:3′-Trimethoxy-4-methyldiphenyl ether (III). 2:5-Dimethoxy-4-bromotoluene (11·6 g.), *m*-methoxyphenol (6·2 g. purified as described by Perkin *et al.* [1926]) and powdered potassium hydroxide (2·8 g.) were dissolved in a little alcohol and the solution heated (bath at 200–220°) for 7 hours. At first alcohol distilled and later a little of the bromo-compound and water. The product was isolated by means of ether and distilled, B.P. 192–195°/2·5 mm. (4·8 g.). The distillate solidified and the substance crystallized from methyl alcohol as stout, colourless prisms, M.P. 72–74° (corr.). (Found: C, 70·1; H, 6·6 %. $C_{16}H_{18}O_4$ requires C, 70·0; H, 6·6 %.)

The *tribromo-derivative* was prepared by the addition of bromine to a moderately concentrated solution of the ether in acetic acid. It crystallized from methyl alcohol in colourless needles, M.P. 130°. (Found: C, 37·5; H, 3·2; Br, 46·7%. $C_{16}H_{15}O_4Br_3$ requires C, 37·6; H, 2·9; Br, 47·0%.)

2:5:3′-Trimethoxy-3-methyldiphenyl ether (IV). This substance was prepared in exactly the same way as the isomeride last described, but starting from 2:5-dimethoxy-3-bromotoluene [Kohn & Steiner, 1931] (11·6 g.). 5 g. of a colourless oil, B.P. 165–166°/0·5 mm. were obtained. (Found: C, 70·1; H, 6·5%.)

The *tribromo-derivative* was obtained under the following conditions. The trimethoxymethyldiphenyl ether (0·1 g.) was dissolved in acetic acid (1·5 ml.) and bromine (0·5 ml.) added gradually with stirring. After 2 min. the product (0·15 g.) was isolated after the addition of aqueous sodium bisulphite. The derivative crystallized from methyl alcohol in colourless needles, M.P. 133° (corr.). (Found: C, 37·4; H, 3·3; Br, 47·2%. $C_{16}H_{15}O_4Br_3$ requires C, 37·6; H, 2·9; Br, 47·0%.)

The characteristic *tetrabromo-derivative* is obtained when an excess of bromine is added to a mixture of the ether with about three times its volume of acetic acid and, if the scale of the operation is so small that the liquid does not become warm as the result of the reaction, the mixture is heated to 50° for 1 min. After allowing to cool, aqueous sodium bisulphite is added and the precipitate collected and crystallized from methyl alcohol, in which the substance is very sparingly soluble, and from ethyl alcohol. It crystallizes in well-shaped, colourless prisms, M.P. 152°. (Found: C, 32·7; H, 2·6; Br, 54·6%. $C_{16}H_{14}O_4Br_4$ requires C, 32·6; H, 2·4; Br, 54·3%.) This tetrabromo-compound was prepared from the product of decomposition of ravenelin trimethyl ether with sodamide and had M.P. 152°, alone or mixed with the synthetic specimen. The crystal forms and solubility relations of the two specimens were also identical. The colourless solution in concentrated sulphuric acid becomes successively pink, yellow, brown and reddish brown on heating.

4:5:3′-Trimethoxy-2-methyldiphenyl ether (V). This was prepared like the isomerides from 3:4-dimethoxy-6-bromotoluene [Heap *et al.* 1927; Erdtmann, 1933] and had B.P. 160–162°/0·2 mm. The distillate crystallized from alcohol, separating as colourless prisms, M.P. 68–69° (corr.). (Found: C, 70·6; H, 6·0%.)

Tetrabromo-derivative. This was prepared by the action of an excess of bromine on a cold solution of the ether in thrice its weight of acetic acid.

The product crystallized from methyl alcohol, in which it was more readily soluble than the isomeride described above, in colourless, elongated prisms, M.P. 115°. (Found: Br, 54·7%. $C_{16}H_{14}O_4Br_4$ requires Br, 54·3%.) It may be noted that considerable depressions of melting-point were observed on mixing the various tribromo- and tetrabromo-derivatives described in this memoir.

Intermediates for the synthesis of 1:4:6-trimethoxy-3-methylxanthone (VI). These intermediates have all been previously described, but the methods of preparation have been improved.

m-Bromotoluquinone [Claus & Jackson, 1888] (80 g., M.P. 93°) was obtained by oxidation of crude 4:6-dibromo-*o*-cresol [Kohn & Segel, 1925] (266 g.) by means of chromic acid (200 g.) in acetic acid (1 litre) and water (700 ml.) at 60° (maximum). The quinone (60 g.) was reduced by means of stannous chloride (100 g., hydrated) in conc. HCl (100 ml.) and water (200 ml.) at 80° (maximum). The quinol separated on cooling and was collected and directly methylated by means of methyl sulphate (100 ml.) and 25% aqueous NaOH in methyl alcohol (200 ml.). After isolation there were obtained 44 g. of 3-bromo-2:5-dimethoxytoluene, B.P. 133°/13 mm.

2-Hydroxy-4-methoxybenzaldoxime [cf. Späth & Klager, 1934] (73 g., M.P. 140°) was obtained from 2-hydroxy-4-methoxybenzaldehyde [Tiemann & Parrisius, 1880; method of A. M. & R. Robinson, 1932] (66 g.) by oximation (hydroxylamine hydrochloride, 50 g.) in pyridine (200 ml.) for 1 hour on the steam-bath. When the oxime (69 g.) was refluxed with acetic anhydride (350 ml.) for 3 hours, an acetyl derivative was produced and this was isolated and hydrolysed by heating with 10% aqueous NaOH (300 ml.) during 15 min. on the steam-bath. On acidification of the solution 2-hydroxy-4-methoxybenzonitrile (44 g., M.P. 179°) separated and was purified by crystallization from aqueous methyl alcohol.

*2-(2′:5′-Dimethoxy-*m*-tolyloxy)-4-methoxybenzoic acid* (related to VIII). Potassium (3 g.) was dissolved in alcohol (30 ml.) and the solution mixed with 2-hydroxy-4-methoxybenzonitrile (11·4 g.), 3-bromo-2:5-dimethoxytoluene (18 g.) and copper bronze (0·3 g.); the whole was then heated at 200° for 8 hours. The neutral products were isolated by means of ether and distilled, giving:

(*a*) 2·5 g., B.P. 105–170°/12 mm. (nearly all at 105–110°), B.P. 218–220°/751 mm. This was identified as 2:5-dimethoxytoluene [cf. Erdtmann, 1933].

(*b*) 2·7 g., B.P. 120–170°/0·15 mm., crystallizing on cooling and separating from methyl alcohol as long, colourless needles, M.P. 96·5°. (Found: C, 66·4; H, 5·6; N, 9·1%. Calc. for $C_9H_9O_2N$: C, 66·3; H, 5·5; N, 8·6%.) This was identified as 2:4-dimethoxybenzonitrile and afforded 2:4-dimethoxybenzoic acid, M.P. 108°, on hydrolysis with baryta.

(*c*) 2·0 g., B.P. 178–188°/0·13 mm., solidifying on cooling and separating from alcohol, light petroleum (B.P. 80–100°) or aqueous acetic acid as short colourless prisms, M.P. 94–95°; analysis indicated that this consisted essentially of 2-cyano-5:2′:5′-trimethoxy-3′-methyldiphenyl ether (VIII), but it was contaminated with a small proportion of a substance of lower carbon content.

(*d*) 0·3 g., B.P. 188–200°/0·13 mm., largely the same nitrile as (*c*).

The formation of 2:4-dimethoxybenzonitrile is the result of a curious reaction and the substance was also obtained for purposes of comparison by methylation of 2-hydroxy-4-methoxybenzonitrile by means of methyl sulphate and 10% aqueous NaOH in methyl alcoholic solution. The purified substance had M.P. 96·5°, alone or mixed with the reaction product mentioned above.

The fractions (*c*) and (*d*) afforded the desired acid on hydrolysis. The cyanotrimethoxymethyldiphenyl ether (100 mg.) was refluxed with barium hydroxide (10 g.) and water (10 ml.) for 12 hours. The cooled solution was acidified with HCl, the precipitate collected and the acid redissolved in aqueous NaOH and reprecipitated. It was then crystallized from alcohol, separating in the form of microscopic, elongated, rhombic prisms, M.P. 208° (corr.) (20 mg.). (Found: C, 64·2; H, 5·9%. $C_{17}H_{18}O_6$ requires C, 64·1; H, 5·7%.)

1:4:6-Trimethoxy-3-methylxanthone (IX). The carboxytrimethoxymethyldiphenyl ether (40 mg.) was boiled with phosphoryl chloride (7 ml.) for 3 min. The deep yellowish brown solution was mixed with water and made alkaline with NaOH, and the solid was collected (30 mg., M.P. 155°). The substance separated from alcohol as tiny rods, M.P. 157° (corr.). (Found: C, 68·0; H, 5·5%. $C_{17}H_{16}O_5$ requires C, 68·0; H, 5·3%.) The melting point was depressed on admixture with *O*-trimethylravenelin, but, although different from this substance, the synthetic product exhibits a general resemblance to it.

The deep yellow solution in conc. HCl gives a yellow precipitate on the addition of a concentrated solution of $FeCl_3$ in the same solvent.

SUMMARY.

The dried mycelia of the fungi *Helminthosporium Ravenelii* Curtis, a plant pathogen parasitizing the grass *Sporobolus indicus* R., and *Helminthosporium turcicum* Passerini, the causative agent of leaf blight disease of maize, *Zea Mays* L., have been shown to contain considerable amounts of a hitherto undescribed methyltrihydroxyxanthone, for which the name ravenelin is proposed. Ravenelin, $C_{14}H_{10}O_5$, has been shown to be 3-methyl-1:4:8-trihydroxyxanthone, and a number of derivatives and breakdown products are described. The optimum cultural conditions for the production of ravenelin by *H. Ravenelii* have been determined.

We desire to express our indebtedness to Mr W. K. Anslow for much technical assistance during the preparation of crude ravenelin.

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CLXXXVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

LI. THE METABOLIC PRODUCTS OF *ASPERGILLUS TERREUS* THOM.

PART II. TWO NEW CHLORINE-CONTAINING MOULD METABOLIC PRODUCTS, GEODIN AND ERDIN.

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IN a previous communication [Raistrick & Smith, 1935] the metabolism at 24° of five strains of *Aspergillus terreus* Thom on the well-known Czapek-Dox solution, which contains KCl as the sole source of chlorine, was described. It was shown that two of the strains, Ac 100 and No. 45, produced a new water-soluble mould metabolic product, terrein, $C_8H_{10}O_3$. From all five strains the metabolism solutions gave, after the glucose was nearly all consumed, precipitates on addition of HCl, the largest amounts being given by the two strains Ac 100 and No. 45. Ether extraction of the acid precipitate from Ac 100 gave citrinin $C_{13}H_{14}O_5$, the crystalline yellow colouring matter first isolated from *Penicillium citrinum* Thom [Hetherington & Raistrick, 1931], whilst extraction of the precipitate from No. 45 gave a crude crystalline material, M.P. 193–196° decomp., the composition of which forms the subject of the present communication. This material consists essentially of a mixture of two new mould metabolic products containing chlorine, for which the names proposed are *geodin*, $C_{17}H_{12}O_7Cl_2$, M.P. 235° (decomp.), and *erdin*, $C_{16}H_{10}O_7Cl_2$, M.P. 211° (decomp.). The properties and some derivatives of these products are described.

Since it is believed that this is the first recorded instance of one of the lower fungi giving rise to metabolic products containing chlorine, it was necessary to prove that these substances are true metabolic products and not artefacts. The metabolism solution shows very strong reducing properties and, since HCl was used for acidification, it was possible that the chlorinated compounds were formed by addition of HCl to some very reactive substance in solution. Even if it were proved that the same compounds could be obtained by using H_2SO_4 for acidification there would still be a doubt, unless it could be shown that the KCl originally present in the culture medium disappears during the course of metabolism. An experiment was therefore carried out in which estimations of chloride in the metabolism solution were made every few days during the incubation period. It was found that, after an initial lag of a few days during which a good mycelial felt was established, the amount of chloride ion in solution diminished at approximately the same rate as the glucose and, after no further utilization of glucose occurred, the amount of chloride in solution was approximately 6 % of the quantity originally present. At this stage addition of H_2SO_4 to the metabolism solution, while removing practically no chloride from the solution, gave a heavy precipitate, identical in appearance with that obtained by using HCl, and containing almost 50% of the chlorine originally present (as KCl) in the culture medium. From the acid precipitate thus obtained, pure samples of geodin and erdin were isolated. There can thus be no doubt that these two substances are true metabolic products. A further confirmation is provided by the fact that neutral solutions of the sodium salts of both geodin and erdin give, on addition of $FeCl_3$, heavy grey precipitates turning brown on standing, a characteristic reaction given also by the metabolism solution of the mould.

It obviously became of interest to test whether brominated and iodinated metabolic products corresponding to geodin and erdin could be isolated from metabolism solutions of *A. terreus*, grown on Czapek-Dox media containing equivalent amounts of KBr or KI in place of KCl. Unfortunately, although practically normal growth was obtained on such media, no metabolic products containing bromine or iodine could be isolated.

It is of interest to note the announcement by Nolan [1934; Hardiman *et al.* 1935] of the isolation of two lichen acids containing chlorine, gangaleoidin, $C_{18}H_{14}O_7Cl_2$, from *Lecanora gangaleoides*, and diploicin, $C_{16}H_{10}O_5Cl_4$, from *Buellia canescens*. Although we have as yet no evidence of structural relationship, the close similarity between the empirical formulae of these lichen acids and of the new mould products is suggestive.

Work is at present in progress on the molecular constitution of geodin and erdin and will be reported in a future communication.

EXPERIMENTAL.

Details of the cultural conditions and of the method of isolation of the crude ether-soluble fraction of the acid precipitate have already been given [Raistrick & Smith, 1935, p. 609].

Chlorine metabolism.

100 flasks of Czapek-Dox solution (glucose, 50 g.: $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water, 1 litre), 350 ml. in each litre conical flask, were sown with a spore suspension of *A. terreus*, strain No. 45, and incubated at 24°. Single flasks were used at intervals for estimation of residual chloride in solution and of apparent residual glucose by polarimeter. Direct estimation of chloride was rendered impossible by the fact that the metabolism solution, after about 14 days' incubation, rapidly reduces $AgNO_3$. Treatment with lead acetate removes interfering substances, but the precipitate carries down some chloride even although the total chloride in the original culture medium is less than that contained in a saturated solution of $PbCl_2$. It was found, however, that norit charcoal will completely remove all interfering substances and does not adsorb any chloride. The metabolism solution, when the glucose has been reduced to about 1%, is dark brown in colour, gives a thick brown precipitate when acidified, reduces $AgNO_3$, $KMnO_4$ or iodine rapidly in the cold and gives a heavy grey precipitate on addition of $FeCl_3$. After treatment in the cold with 4–5% of norit the solution is colourless, gives no precipitate on acidification, does not reduce $AgNO_3$, reduces $KMnO_4$ only slowly and gives no reaction with $FeCl_3$. The minimum amount of norit required is 4 g. per 100 ml. solution until the glucose is reduced to about 0·4% and thereafter 5 g. per 100 ml. It was shown by tests on Czapek-Dox solution that norit does not adsorb any of the chloride in the medium but that the solution extracts a very small amount of chloride from the norit, equivalent to 0·0016 g. KCl per 5 g. norit. This was, of course, allowed for in all estimations.

The actual method of estimation was to shake 100 ml. of metabolism solution with 5 g. of norit for 10 min. in the cold, filter and collect exactly 85 ml. of clear filtrate, add HNO_3 and standard $AgNO_3$ solution and titrate the excess silver by the Volhard method. The results are shown in Table 1.

Table I.

Days' incubation	Apparent residual glucose by polarimeter %	Residual chloride as KCl %
0	5·00	0·0497
9	2·85	0·0420
12	1·77	0·0338
14	1·50	0·0323
17	0·85	0·0161
19	0·23	0·0072
21	0·81	0·0116
24	0·35	0·0057
26*	0·37	0·0034
Acid filtrate		0·0028

* Average of 91 flasks.

The figures take no account of evaporation of the medium during incubation. Actually, at the end of the period of incubation, the volume of filtered metabolism solution from 100 flasks, originally containing 35 litres of medium is, on the average, 31 litres.

There was some irregularity amongst individual flasks, and the figures obtained from analyses of the contents of single flasks do not give smooth curves for consumption of glucose and chloride. They show, however, that after an initial period during which the mycelial felt is being established the chloride disappears at approximately the same relative rate as the glucose.

After 26 days' incubation the contents of the remaining 91 flasks were filtered and the filtrate, 28 litres, divided into two equal portions, one being made acid to Congo red by addition of 75 ml. concentrated HCl, and the other acidified with an equivalent amount (375 ml.) of $2N$ H_2SO_4. Flocculent brown precipitates were formed in both solutions, the two being identical in appearance. The precipitates were filtered off, washed with water until free from acid and chloride and dried *in vacuo*. The two were identical in weight, 15·5 g. Residual chloride was estimated in the filtrate obtained after acidification with H_2SO_4. As seen from Table I it was slightly less than before acidification but the difference is very small compared with the total amount of chlorine in the precipitate. Estimation of chlorine in the crude H_2SO_4 precipitate gave 11·4 %. The chloride originally present in the culture medium is therefore accounted for as follows:

0·0497 % KCl in 15·925 litres medium (half of 91 flasks) = 3·764 g. Cl.
0·0034 % KCl in 14 litres metabolism solution = 0·226 g. Cl = 6 % original.
11·4 % Cl in 15·5 g. acid precipitate = 1·767 g. Cl = 47 % original.
Unaccounted for = 1·771 g. Cl = 47 % original.

The two acid precipitates, obtained by acidification with HCl and H_2SO_4 respectively, were exhaustively extracted with ether, side by side in Soxhlet extractors. The crystalline material collecting in the flasks was filtered off at intervals and the ether mother-liquors were evaporated to obtain further crops until only tarry matter remained. The total amounts of crude crystalline material obtained were, respectively, *ex* HCl precipitate 7·25 g.; *ex* H_2SO_4 precipitate 7·28 g. The two lots were apparently identical and both were shown, by fractional crystallization as described below, to consist essentially of geodin and erdin.

Fractionation of crude crystalline material.

Fractional extraction with ether of the combined acid precipitates from several 100-flask experiments resulted in a partial separation of the two substances. The more soluble fractions melted at about 195° with blackening and vigorous evolution of gas and gave a colourless crystalline sublimate when further heated to 230°. The first crops obtained from the later extracts, representing the less soluble material, melted at about 220° with evolution of gas but did not blacken or give any sublimate on further heating to 250°. All the fractions were pale buff in colour and dissolved readily in dilute NaOH to give bright yellow solutions which could be back-titrated quite sharply with HCl. The various fractions were sorted according to the optical rotations of the neutral solutions. For the fractions of low M.P. $[\alpha]_{5461}$ varied from $+7°$ to $+50°$, whilst fractions which showed no blackening on heating gave $[\alpha]_{5461}$ approximately $+70°$. Calculations from the specific rotations, under these conditions, of geodin ($+72°$) and erdin (zero) showed that the two substances were present in approximately equal amounts in the crude material. Attempts at further fractionation with ether were unsuccessful, the two substances having almost equal solubilities, and hence the preliminary separation depended on the relative solubilities in presence of the brown tarry material which was always extracted along with the crystalline products. A fairly clean separation was effected by using $CHCl_3$, in which geodin is readily soluble and erdin very sparingly soluble. Each crude fraction was boiled with $CHCl_3$, 10 ml. for each 1 g. of geodin present, the solutions being filtered hot and diluted with 2 vol. of ether. On cooling, geodin separated in good yield in rosettes of fine needles, pale brown in colour, M.P. 225–230° (decomp.). Recrystallization from ethyl acetate gave fine needles, still brownish, M.P. 235° (decomp.), and further recrystallization did not raise the M.P. By sublimation in a high vacuum at 200° the trace of brown colouring matter was eliminated, the sublimate consisting of a micro-crystalline powder of a clear pale yellow colour, M.P. 235° with evolution of gas but without any sublimate.

The residues from the $CHCl_3$ extractions were crystallized several times from ethyl acetate and erdin was thus obtained as pale yellow needles, M.P. 211°, blackening with evolution of gas and giving a sublimate in the form of long colourless needles when heated to 230°.

It was shown, by fractional crystallization of the crude crystalline material from ethyl acetate, that geodin and erdin do not owe their chlorine content to the use of $CHCl_3$ as solvent, but this method is much more tedious than the one involving the use of $CHCl_3$.

PROPERTIES AND ANALYSES OF THE NEW METABOLIC PRODUCTS.

All analyses except OCH_3 estimations were carried out by Schoeller (Berlin). Determinations of mol. wt. by the cryoscopic method were carried out by Dr A. E. Oxford, to whom we tender our best thanks.

Geodin.

Geodin, $C_{17}H_{12}O_7Cl_2$, crystallizes from a mixture of 1 vol. $CHCl_3$ and 2 vol. ether as pale yellow, fine needles, M.P. 235°, with evolution of gas but without blackening or the formation of a sublimate. (Found: (*a*) on sample *ex* HCl precipitate, C, 51·10, 51·01; H, 3·09, 3·00; Cl, 17·61, 17·80; OCH_3, 15·55, 15·87%. Mol. wt. cryoscopic in dioxan, 380; (*b*) on sample *ex* H_2SO_4 precipitate, C, 51·10; H, 3·00; Cl, 17·46%. $C_{15}H_6O_5Cl_2(OCH_3)_2$ requires C, 51·13; H, 3·03; Cl, 17·77;

$2OCH_3$, 15·55 %. Mol. wt. 399.) Optical rotation: in $CHCl_3$ ($c=0{\cdot}8$), $[\alpha]^{20°}_{5461} + 179°$, $[\alpha]^{20°}_{5790} + 149°$.

Geodin is insoluble in water or light petroleum, very sparingly soluble in benzene or ether, somewhat more soluble in alcohol or ethyl acetate and dissolves readily in chloroform, acetone or dioxan. The solution in alcohol gives on addition of $FeCl_3$ solution a dirty green colour changing to brown, whilst a neutral solution of the sodium salt gives a heavy brownish grey precipitate. The substance dissolves readily in $NaHCO_3$ solution.

Titration. If dissolved in a slight excess of $N/10$ NaOH and the solution immediately back-titrated with $N/10$ HCl, geodin behaves as a dibasic acid. Equivalent thus found 198; 199. On long standing, or on heating with excess of alkali, decomposition takes place. The neutral solution of the disodium salt has $[\alpha]^{20°}_{5461} + 72{\cdot}4°$, $[\alpha]^{20°}_{5790} + 65{\cdot}6°$.

Identification of alkoxyl groups. 1 g. of geodin was dealkylated by heating with HI and the gaseous products were passed into freshly distilled dimethylaniline. Colourless plates (1·03 g.) were rapidly deposited and, at the end of the reaction, were filtered off, washed with ether and recrystallized from absolute alcohol. (Found: I, 48·1 %. $C_6H_5.N(CH_3)_3I$ requires I, 48·25 %.) The corresponding compound $C_6H_5.N(CH_3)_2.C_2H_5I$ is not crystalline and requires I, 45·8 %. Hence the alkoxyl groups are methoxyl.

Action of diazomethane on geodin. Geodin (1 g.) was suspended in dry ether and treated with an excess of ethereal diazomethane. There was vigorous evolution of nitrogen and the mixture turned bright pink. At the end of the reaction there was present a considerable amount of red crystalline solid. This was filtered off and washed with ether; wt. 0·93 g., M.P. 151° decomp. A further 0·03 g. was obtained by evaporation of the ethereal mother-liquor. Under the microscope the material appeared as colourless crystals with a small amount of red dye adsorbed rather than as red crystals. By short boiling in MeOH solution the red colour was destroyed and, on cooling, the solution deposited fine colourless needles, M.P. 151° decomp.; insol. in $NaHCO_3$. (Found: C, 50·31, 50·35; H, 3·51, 3·56; N, 6·31, 6·27; Cl, 15·35, 15·64; OCH_3, 20·33, 20·37 %. Mol. wt. cryoscopic in nitrobenzene, 419. $C_{16}H_7O_4N_2Cl_2(OCH)_3$ requires C, 50·10; H, 3·54; N, 6·15; Cl, 15·58; $3OCH_3$, 20·43 %. Mol. wt. 455). Treatment with diazomethane therefore results in the introduction of one new methyl group and the addition of one molecule of CH_2N_2, presumably at a double bond. The substance is optically active, having, in benzene ($c=0{\cdot}9$) $[\alpha]^{22°}_{5461} - 58°$, $[\alpha]^{22°}_{5790} - 44°$.

Catalytic reduction; dihydrogeodin. Geodin (1 g.), dissolved in cold absolute alcohol (250 ml.) $[\alpha]^{25°}_{5461} + 152°$ was shaken with hydrogen in presence of a catalyst prepared from palladium chloride (0·5 g.) and charcoal (1·5 g.). The uptake of gas was very rapid, being complete in 30 sec., and the volume absorbed corresponded with the entry of two hydrogen atoms into the molecule. The catalyst was filtered off and the alcoholic solution, now optically inactive, was evaporated *in vacuo* to 40 ml. On addition of 2 vol. of H_2O dihydrogeodin separated as fine needles of a bright yellow colour, M.P. 216–217° decomp. raised to 229° *ex* ether-light petroleum; yield 0·84 g. (Found: C, 51·05, 50·76; H, 3·72, 3·70; Cl, 17·59, 17·45; OCH_3, 15·46 %. $C_{17}H_{14}O_7Cl_2$ requires C, 50·87; H, 3·52; Cl, 17·68; $2OCH_3$, 15·47 %.) Dihydrogeodin is readily soluble in alcohol, ether, ethyl acetate or acetone and almost insoluble in H_2O, $CHCl_3$, C_6H_6 or light petroleum. Its alcoholic solution gives an olive-green colour with $FeCl_3$.

Acetylation of geodin. Attempts to prepare an acetyl compound of geodin have so far been unsuccessful. Treatment with acetic anhydride and pyridine in the cold led to dark brown tarry products. Heating geodin (1 g.) with a mixture of acetic anhydride (4 ml.) glacial acetic acid (6 ml.) and anhydrous sodium acetate (2 g.) at 140–150° for 20 min. gave a mixture of substances which were apparently acetyl derivatives of breakdown products.

Erdin.

Erdin, $C_{16}H_{10}O_7Cl_2$, was obtained by crystallization from ethyl acetate as fine yellow needles, M.P. 211°, with blackening and vigorous evolution of gas. Frothing increases as the temperature is raised and at 225–230° a sublimate collects in the upper part of the M.P. tube in the form of long colourless needles.

Erdin clings most tenaciously to the solvents used for its purification and these cannot be removed completely even in a high vacuum. Hence the analyses submitted below are not in themselves sufficiently conclusive to establish beyond doubt the empirical formula for erdin. Fortunately the crystalline product obtained by the action of diazomethane on erdin (see below) was obtained free from solvent and the results of its analysis leave no room for doubt that erdin itself has the empirical formula $C_{16}H_{10}O_7Cl_2$.

Thus a sample of erdin recrystallized from ethyl acetate containing a little ethyl alcohol lost no weight on heating in nitrogen at 110°, but apparently contained 0·5 mol. of ethyl alcohol. (Found: C, 50·08, 50·06; H, 3·31, 3·29; Cl, 17·20, 17·30; OCH_3, 11·5, 11·4%. Mol. wt. cryoscopic in dioxan, 297, 292. $C_{15}H_7O_6Cl_2(OCH_3)$, $0{\cdot}5C_2H_5OH$ requires C, 50·00; H, 3·21; Cl, 17·38; OCH_3 (1·5 groups) 11·4%. Mol. wt. if completely dissociated in dioxan, 272. $C_{15}H_7O_6Cl_2(OCH_3)$ requires C, 49·87; H, 2·62; Cl, 18·42; $1OCH_3$, 8·06%. Mol. wt. 385.)

Erdin crystallizes from a mixture of 1 vol. dioxan and 2 vol. H_2O in beautiful yellow rectangular plates containing 1 mol. dioxan and 2 mol. H_2O of crystallization, M.P. 193° decomp. (Found on air-dried sample: C, 47·31, 47·46; H, 4·31, 4·30; Cl, 14·03, 13·80%. $C_{16}H_{10}O_7Cl_2$, $2H_2O$, $C_4H_8O_2$ requires C, 47·14; H, 4·35; Cl, 13·93%.) OCH_3 could not be estimated since dioxan is decomposed by HI to give an indeterminate amount of C_2H_5I. A sample dried to constant weight at 135° lost 23·70% and had M.P. 210–211°. Calculated loss of $1{\cdot}5H_2O+C_4H_8O_2$, 22·6%. Analysis of the dried product showed that 0·5 mol. H_2O was still retained. (Found: C, 49·00, 49·01; H, 2·88, 2·84; Cl, 17·82, 17·84; OCH_3, 7·83, 7·96%. $C_{15}H_7O_6Cl_2(OCH_3)$, $0{\cdot}5\,H_2O$ requires C, 48·73; H, 2·81; Cl, 18·00; $1OCH_3$, 7·87%.) Further heating at 150° in a high vacuum resulted in no further loss. (Found: C, 48·98, 49·13; H, 2·82, 2·71; Cl, 17·93, 17·96%.) At higher temperatures obvious decomposition occurred. The substance could not be sublimed unchanged in the highest vacuum attainable with a mercury diffusion pump.

Titration. Erdin titrates in the same way as geodin, reacting as a dibasic acid and decomposing slowly in presence of excess of alkali; equiv. wt. 192. $C_{16}H_{12}O_7Cl_2$, $0{\cdot}5H_2O$, as a dibasic acid requires 197.

Optical rotation. Both erdin itself and its disodium salt are optically inactive.

The solubilities of erdin are closely similar to those of geodin except that it is only very slightly soluble in $CHCl_3$.* It also shows reactions with $FeCl_3$ identical with those given by geodin, giving a dirty green colour turning brown in alcoholic solution, and a heavy grey precipitate as the sodium salt in H_2O. Like geodin also erdin dissolves readily in $NaHCO_3$ solution.

Action of diazomethane on erdin. Erdin (1 g.) was suspended in dry ether and treated with an excess of ethereal diazomethane. The reaction proceeded exactly as with geodin, the product being bright pink and almost insoluble in ether; yield 0·94 g.; M.P. 154°; insol. in $NaHCO_3$. By crystallization from MeOH colourless needles were obtained, M.P. 154° (decomp.), mixed M.P. 142–144° with the corresponding product from geodin (M.P. 151°). (Found: C, 50·15, 50·32; H, 3·62, 3·53; N, 6·21, 6·22; Cl, 15·53, 15·69; OCH_3, 20·45, 20·32 %. Mol. wt. cryoscopic in nitro-benzene 435. $C_{16}H_7O_4N_2Cl_2(OCH_3)_3$ requires C, 50·10; H, 3·54; N, 6·15; Cl, 15·58; $3OCH_3$, 20·43 %. Mol. wt. 455.) By the action of diazomethane, therefore, two new methoxyl groups have been introduced and one molecule of CH_2N_2 added on, the product having the same empirical formula and containing the same number of methoxyl groups as the corresponding product from geodin. Unlike the latter, however, the product from erdin is optically inactive. This fact, together with the depression in M.P. noted above, leaves no doubt that the substances are not identical.

Catalytic reduction; *dihydroerdin*. Erdin (1 g.) dissolved in absolute alcohol (100 ml.) was shaken with hydrogen in presence of a palladium-charcoal catalyst. Hydrogenation proceeded exactly as in the case of geodin, 2 atoms of hydrogen being taken up in approximately 30 sec. The catalyst was filtered off, the solution evaporated *in vacuo* to 40 ml. and water added till the solution was faintly turbid. The product crystallized in bright yellow needles (0·86 g.), M.P. 240° with evolution of gas and formation of a colourless crystalline sublimate resembling that obtained from erdin itself. For analysis the material was dried at 80° over $CaCl_2$ *in vacuo*, there being no further loss in weight at 110° *in vacuo*. (Found: C, 48·90, 48·92; H, 3·38, 3·27; Cl, 17·80, 17·77 %. $C_{16}H_{12}O_7Cl_2$ requires C, 49·61; H, 3·13; Cl, 18·32 %. $C_{16}H_{12}O_7Cl_2, 0{\cdot}5H_2O$ requires C, 48·48; H, 3·31; Cl, 17·90 %.) Dihydroerdin, like the parent substance, erdin, thus contains 0·5 mol. of water of crystallization which cannot be removed even by intensive drying.

Dihydroerdin, which is optically inactive, is readily soluble in ether, alcohol, ethyl acetate or acetone, and practically insoluble in H_2O, light petroleum, $CHCl_3$; or C_6H_6. In alcoholic solution it gives an olive-green colour with $FeCl_3$. 0·5 g. treated with ethereal diazomethane gave a thick yellow oil which could not be crystallized.

Acetylation. On acetylation of erdin with acetic anhydride, glacial acetic acid and anhydrous sodium acetate, a mixture of substances was obtained which has not yet been satisfactorily fractionated.

EXPERIMENTS ON CULTURE MEDIA CONTAINING BROMIDE AND IODIDE.

Experiments were carried out with a view to finding whether *A. terreus* can utilize bromide and iodide and build up metabolic products similar to geodin and erdin but containing bromine or iodine in place of chlorine.

35 litres of a modified Czapek-Dox solution, containing 28 g. KBr in place of 17·5 g. KCl, were distributed in 100 flasks in the usual way and sown with a spore suspension of *A. terreus* No. 45. Growth was rapid and very similar to that on the normal medium. Attempts to estimate residual bromide were unsuccessful, the method used for estimation of chloride being inapplicable owing to the norit adsorbing an appreciable amount of bromide from solution. After 24 days the residual glucose was 0·37 % (polarimeter), the solution being dark brown and giving a definite precipitate on addition of mineral acid. The contents of all the flasks were therefore filtered and the clear solution acidified by addition of 700 ml. of $2N$ H_2SO_4. The precipitate was filtered off, thoroughly washed and dried *in vacuo*. It was thus obtained as a brown powder, 10·7 g. No halogen could be detected in the dry precipitate by the usual tests. Extraction with ether gave a considerable amount of tar and a small quantity (0·85 g.) of brownish crystalline material, M.P. 200–210° decomp. Tests for the presence of bromine in this material were negative.

In a second experiment the KCl of Czapek-Dox solution was substituted by an equivalent quantity (39 g.) of KI. Growth was rapid and fairly normal, there being somewhat more variation amongst individual flasks than when the normal medium was used. After 24 days the glucose in solution was reduced to 0·31 %, and the contents of all the flasks were then worked up. The dry acid precipitate, 10 g., which was almost black and carbonaceous, contained no iodine and gave only a small amount of tar on extraction with ether.

SUMMARY.

A strain of *Aspergillus terreus* Thom, when grown at 24° on Czapek-Dox solution containing KCl as sole source of chlorine, gives rise to two hitherto undescribed, chlorine-containing metabolic products for which the names *geodin*, $C_{17}H_{12}O_7Cl_2$, and *erdin*, $C_{16}H_{10}O_7Cl_2$, are proposed. Methods of isolation and certain derivatives are described. The substitution of KBr or KI for KCl in the Czapek-Dox medium did not result in the isolation of brominated or iodinated metabolic products, although practically normal growth of the mould was obtained.

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CLXXXIX. BACTERIOLOGICAL AND BIOCHEMICAL RELATIONSHIPS IN THE PYOCYANEUS-FLUORESCENS GROUP.

I. THE CHROMOGENIC FUNCTION IN RELATION TO CLASSIFICATION.

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In the system of bacterial classification proposed by a committee of the Society of American Bacteriologists [Bergey, 1934], the very closely related green fluorescent bacteria occurring chiefly in soil and water are grouped together as the genus *Pseudomonas*. In common with most other bacterial characteristics pigmentation has, from time to time, been considered a variable cultural factor. According to Gessard [1890], a given strain of *B. pyocyaneus* may, by suitable cultural methods, be induced to form the fluorescent pigment alone, pyocyanine alone or the two pigments simultaneously. Charrin & Phisalix [1892] record the persistent abolition of chromogenic function with *B. pyocyaneus*. More recently, Baerthlein [1918] has obtained, by selection of colonies, six variants from a single strain of *B. pyocyaneus*. His original strain gave two types of colony, (1) dry, opaque colonies, containing short, stumpy cells, (2) moist, translucent colonies of long slender bacteria. Each of these gave three further variants (*a*) in colourless colonies, (*b*) in pale green colonies, producing the green fluorescent pigment but not the blue pyocyanine, and (*c*) in deeper green colonies, producing both fluorescent pigment and pyocyanine. From this it would seem a matter of extreme difficulty, if not actually impossible, to decide definitely whether a given bacillus is one of the fluorescent type or merely a strain of *B. pyocyaneus* which has lost its typical property of producing the blue pyocyanine, or whether it belongs to the group at all.[1] Previous observations (unpublished) in connexion with another problem have led the author to believe that pigment production in this group might probably be much more constant than has hitherto been supposed. The investigations described in this paper were undertaken in an endeavour to ascertain to what extent the chromogenic function can be regarded as invariable and of sufficient fundamental importance to act as the basis for the classification of these bacteria.

EXPERIMENTAL.

(1) *Bacteriological examination of fluorescent bacteria.*

The bacteria used (100 strains) were isolated as follows: 25 strains from water (River Trent, canal water, reservoirs, etc.) 25 from fresh faeces (man, animal, fowl), 25 from soil and the remainder from various widely differing sources; in every case growth on agar slants was accompanied by diffusion of a greenish fluorescence through the medium, this being the criterion for inclusion amongst the stock strains. In addition, pure cultures of *B. pyocyaneus*, *B. fluorescens liquefaciens* and *B. fluorescens non-liquefaciens* were obtained from the National Collection of Type Cultures, Lister Institute, London (Catalogue Nos. 1999, 964, 3247 respectively). The standard bacteriological examinations were conducted in each case from 24-hour cultures on nutrient agar slants, and are briefly summarized:

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[1] A comprehensive survey of the literature is included in a thesis, "The Metabolic Pigments of the Pyocyaneus-Fluorescens Group," to be presented by the author for the degree of Ph.D. (Lond.), 1936.

The cells, in every case, were small rods 0·5 × 2·0 μ; the variation in size was remarkably small, and rods longer than 2·0 μ were found in only 7 strains.

All were actively motile, but in several cases (chiefly from acid clay soil) the staining of flagella was unsuccessful. In the other strains, staining by the method of Gray [1926] showed 1–3 polar flagella.

Endospores and capsules were never encountered.

The strains were invariably Gram-negative.

Both liquefaction of gelatin and formation of indole occurred in about 70% of the strains. This figure is considerably higher than that of Tanner [1918] who, however, worked with fluorescent bacteria solely from water.

Approximately the same percentage of strains was found to be able to grow under anaerobic conditions in presence of nitrate or nitrite. This facultative anaerobic growth has been described very thoroughly in the case of *B. pyocyaneus* by Quastel *et al.* [1925].

No gas was obtained from carbohydrates, but in many cases a small amount of acid was produced from glucose.

Investigations on the pathogenicity of the various strains were not attempted.

From a purely bacteriological standpoint the above examination shows that these strains form a homogeneous group of very closely related individuals, and on these characteristics alone little more can be said concerning their classification.

(2) *Cultural requirements for pigment production.*

Investigations on this much-debated subject [Gessard, 1890; 1892; Noesske, 1897; Kuester, 1899; Jordan, 1899, 1, 2; Sullivan, 1905; *et al.*] seemed to have culminated with the painstaking researches of Benecke [1907], who showed that the essentials for production of fluorescent pigment were Mg, phosphate, sulphate, and a very small amount of K^+ ions, together with suitable sources of C and N. The subject was again reopened by Georgia & Poe [1931; 1932] and by Robinson [1932]; these workers agree that in synthetic media requirements for production of fluorescence are available C and N, with Mg and phosphate. For the present work this conclusion has been accepted, and attention has been directed towards the discovery of media by means of which the stock strain of typical *B. pyocyaneus* might be induced to form (*a*) both pyocyanine and fluorescent pigment, (*b*) pyocyanine alone, (*c*) fluorescent pigment alone, (*d*) neither pigment. The media used by Gessard [1890; 1891] were tried, but with the exception of the glycerol-peptone-agar described below they did not give altogether consistent results. It was thought better to use, wherever possible, media of definite chemical composition in order that the exact conditions in any particular case might be repeated. Experiments conducted on many different media, involving numerous permutations, gave the following as optimum conditions.

(*a*) The presence of peptone and of glycerol seemed to favour production of pyocyanine, whilst gelatin caused considerable formation of fluorescent pigment.

Ordinary double-strength broth, with the addition of 1 % glycerol, gave abundant production of both pigments. (No attempt was made to find a definite chemical medium here, since production of both pigments simultaneously was not required in connexion with the classification.)

(*b*) On the following glycerol-peptone-agar medium [Gessard, 1891]:

Medium A.		
	Glycerol	5 %
	Peptone	2 %
	Agar	3 %

which should be neutral to phenolphthalein and sterilized by autoclaving for 15 min. at 10 lb. pressure, there was profuse formation of pyocyanine but little fluorescent pigment. On slightly increasing the peptone constituent (2·5 %), fluorescence was undetectable. No chemical medium tried gave pyocyanine at all comparable in quantity with that obtained on this medium. Various makes of peptone and agar were used, the best results being obtained from "Difco" Brand Bacto-Peptone and B.D.H. powdered agar; these products were used exclusively in the later work.

(*c*) The medium recommended by Georgia and Poe [1931]:

Asparagine	0·3 %
K_2HPO_4	0·05 %
$MgSO_4, 7H_2O$ ...	0·05 %
Distilled water	

proved to be satisfactory in inhibiting pyocyanine production, whilst at the same time giving good yields of fluorescent pigment. In view of proposed attempts on the isolation of the green fluorescent pigment, and the enormous quantities of culture medium required, it was decided to find if possible a less expensive substitute for asparagine. After the trial of many C and N sources, the following liquid medium was evolved which gave very intense green pigment production and no pyocyanine detectable by extraction with chloroform:

Medium B:		
	NH_4NO_3	0·1 %
	K_2HPO_4	0·025 %
	$MgSO_4, 7H_2O$ ...	0·025 %
	Distilled water	

This medium was sterilized by steaming in a Koch sterilizer at 100° during 1 hour on each of three successive days, after which 0·1 % freshly-boiled alcohol was added by means of a sterile pipette. Better results were obtained by the use of larger quantities than the usual culture tubes. Two-litre quantities of the medium, contained in plugged 2500 ml. flat-bottomed flasks, were inoculated from 24-hour agar slant cultures of *B. pyocyaneus* and incubated at 25°. After 12 days the medium showed a green fluorescence and a cloudiness due to bacterial growth. On transferring the contents of one of the flasks to a 2000 ml. beaker, adding 5 ml. of a concentrated NaOH solution and warming to about 85°, a thick white coagulum of protein formed on the surface of the liquid, which now was transparent and showed a bright green fluorescence.

(*d*) If, from medium B, Mg or phosphate is omitted, growth still occurs and a cloudiness develops throughout the medium, but no colour is obtained. Similarly, if a trace of some heavy metal salt be added there is complete inhibition of all pigment formation, although growth proceeds. The minute amounts of salts present in tap-water will completely prevent the development of any colour if such water is used instead of distilled water in preparing the culture medium.

Medium C:	NH_4NO_3	0·1 %
	K_2HPO_4	0·025 %
	$MgSO_4, 7H_2O$ ...	0·025 %
	$FeSO_4, 7H_2O$ ...	0·0001 %
	EtOH	0·1 %.

B. fluorescens liquefaciens and *B. fluorescens non-liquefaciens* were both cultured on the above media A, B, C, but the results obtained were widely different from those described for *B. pyocyaneus*. In no case was growth accompanied by any pigmentation, although on broth and in the asparagine medium a greenish fluorescence developed.

Each of the strains isolated from natural sources was subcultured three times at intervals of 7 days on each of the three media A, B, C, and incubated at 25°. They were found to fall, with respect to their chromogenic functions, into two sharply defined groups corresponding to (*a*) *B. pyocyaneus*, (*b*) *B. fluorescens*.

(3) *Constancy of the chromogenic function in* B. pyocyaneus *and* B. fluorescens.

According to Sullivan [1905] varieties of *B. pyocyaneus* producing pyocyanine alone and fluorescent pigment alone cannot be made to take up the production of the other pigment, although a variety producing both pigments may be made to exhibit either or both. This view is somewhat at variance with Gessard's [1890] results already quoted, and with the report of Meader *et al.* [1925] that all bacteria isolated by them from natural sources gave definite production of pyocyanine after subculture on Gessard's glycerol-peptone-agar.

During the past 18 months, intensive selective cultivation in this group has been carried out by the author. The stock strain of *B. pyocyaneus* has been subcultured repeatedly at intervals of 7 days upon each of the media A, B and C and incubated at 25°. It was thought reasonable to assume that if pigment production were a readily variable feature such treatment would modify to some extent this characteristic of the organism. At the end of the time, however, there appeared to have been no change in this respect, the other chromogenic features being readily recovered by subculture on the appropriate medium, thus substantiating the results obtained by Gessard [1890]. Spontaneous degeneration with loss of chromogenic power [see Jordan, 1899, 1, 2; *et al.*] has not been encountered in this investigation.

These experiments were carried out in addition with *B. fluorescens* and also with certain selected natural strains (No. 3 from canal water; No. 37 from human faeces; No. 69 from uncultivated soil; No. 81 from snow), but no permanent change in the colour-producing properties was ever observed. *B. fluorescens* and Strains 69, 81 could not at any time be induced to form pyocyanine, whereas Strains 3, 37 behaved exactly as the stock strain of *B. pyocyaneus*. In view of these results, the observation suggests itself that the bacteria used by Meader *et al.* [1925] were, without exception, strains of *B. pyocyaneus*, especially as they report that their organisms were isolated from environment in close proximity to man.

DISCUSSION.

The very unsatisfactory present state of bacterial classification may be accounted for almost entirely by the ever increasing records of variation. Biological characters have, in various instances, been supplemented for purposes of classification by consideration of purely chemical features. The genus *Lacto-*

bacillus, for example, is composed of organisms producing a common metabolic product, lactic acid, on carbohydrate media. The formation of a metabolic product, whether it ultimately serves some useful purpose or whether it is merely a waste product, is a relatively constant property and as such is more especially suited to act as the basis for the classification of the organism producing it. So distinctive a property as the production of a diffusible, green fluorescent pigment in the culture medium, would seem a very convenient basis for classification of the responsible organism, subject to the provisions that, firstly, the green pigment is of the same chemical composition in each case; secondly, the organisms are not widely differentiated by their morphological and cultural characteristics: and thirdly, the chromogenic function is sufficiently invariable. Emphasis is placed on the second point, since certain fluorescent organisms of the genus *Phytomonas* are almost identical morphologically and culturally with those of the genus *Pseudomonas*. Even should subsequent research indicate the identity of the green pigment from both groups, the differentiation of the two genera would perhaps still be satisfactorily accomplished on the pathogenicity for plants displayed by the former organisms. There still remains the possibility that the *Phytomonas* and *Pseudomonas* organisms would be better considered as distinct species of a genus whose generic characteristic is the production of a diffusible, green fluorescent pigment. In the present paper, the work has been concerned with the second and third of the points outlined. An investigation on the isolation of the green fluorescent pigment, and the comparison of its composition in a number of selected bacteria is nearing completion. The results, which show the pigment to have an identical composition in the cases examined, will be communicated as a second part to this paper.

The allocation, then, of the green fluorescent bacteria in a single genus (*Pseudomonas*) is strongly supported by definite biochemical evidence. Whilst it is not suggested that the chromogenic function shall serve as more than the generic character, there remains the evidence that within the group the media quoted appear to differentiate between two constant forms:

(1) *B. pyocyaneus* (*Pseudomonas aeruginosa* (Schröter) Migula).
(2) *B. fluorescens* (*Pseudomonas fluorescens* (Flügge) Migula).

The conclusion that the fluorescent group of bacteria contained these two constant forms had been reached on different evidence by various workers [Ruzicka, 1899; Niederkorn, 1900], many years before the introduction of the genus *Pseudomonas*.

Whether the chromogenic function really provides a basis for the further differentiation into species is a problem which must, for the present, remain undecided. The possibility of the discovery of other media giving a finer separation of species within the genus cannot be overlooked.

SUMMARY.

1. Evidence has been obtained for the classification of the pyocyaneus-fluorescens group of bacteria into a single genus on the basis of their chromogenic function.

2. Media have been elaborated upon which a very marked constancy in pigment production is exhibited by these bacteria.

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CXC. STUDIES UPON THE MODE OF ACTION OF VITAMIN D.

I. INVESTIGATIONS UPON THE PHOSPHORUS COMPOUNDS IN MUSCLES, LIVER AND KIDNEYS AS INFLUENCED BY DIFFERENT LEVELS OF VITAMIN D AND PHOSPHORUS IN THE DIET.

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In recent years evidence has been obtained that vitamin D may play an important part in intermediary P metabolism. Thus in deficiency disease change in concentration of P compounds has been demonstrated not only in blood but also in tissue. The question as to whether vitamin D acts in a specific way on intermediary P metabolism and, if so, how it acts, is however far from solved, as will be seen from a short review of earlier investigations in this field.

Sharpe [1922] estimated the phospholipins in livers from normal and rachitic dogs. This work is perhaps open to the criticism that the livers had been preserved in formalin. This substance is often contaminated with formic acid, and it is noteworthy that the phospholipins are unstable in the presence of acids. Nevertheless it is difficult to understand how this could cause the striking decrease in concentration of phospholipins which he found in the rachitic dogs' livers. Cole & Koch [1931] estimated the acid-insoluble P (i.e. the protein and phospholipin-P in one fraction) in muscles from normal and from rachitic rats on McCollum diet. They found a reduction of the "muscle-P", and conclude that "the results suggest that the irradiated ergosterol simply hastened the transfer of muscle-P to bone formation at the expense of this type of muscle-P. However, the quantitative handling of this fraction was so difficult that the difference may not be significant". The total lipin- and protein-P in this series of investigations was sometimes lower than the usual P content of the muscle lipin alone, and it seems desirable to repeat the experiments before such an important suggestion can be accepted. McGowan [1933] suggests that vitamin D acts by setting free "nascent phosphoric acid" from the phospholipins of the liver. The experimental evidence for this mode of action of vitamin D is not given. Kay [1932] estimated inorganic P and acid-soluble P in liver and kidneys from normal and rachitic rats—partly using Steenbock's, partly McCollum's diet. The results, as Kay points out, are not very easy to interpret. However, they give the impression that the phosphoric esters are reduced to some extent in rickets. The clearest results were those of Skill & Kay [1934] in beryllium rickets where a great reduction was seen both in inorganic and in ester P. Cole & Koch also estimated the acid-soluble fraction of muscle-P; there was, however, no marked difference between normal and rachitic animals. Hentschel & Zoeller [1927] investigated total and inorganic P in the muscles of normal rats and rats on McCollum's diet, Sherman-Pappenheimer's diet and a modified Steenbock diet. They failed to find any regular alteration in the total P, but more often the inorganic P was greatly reduced, increasing towards normal on antirachitic treatment. They state, however, that the rachitic rat muscles generally contained more water than those from normal animals.

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In addition to the well-known reduction of inorganic P in rickets other fractions of the blood-P are also stated to be affected. Skill & Kay [1934], in the experiments mentioned above, found a reduction of phosphoric esters in red blood cells in rachitic rats, particularly striking in the severe beryllium rickets. The withdrawal of beryllium from the diet was followed by an increase towards normal. Bakwin *et al.* [1932] found that in rachitic infants the fraction of the blood P easily hydrolysed by mineral acids—pyrophosphate—was reduced from 14 to 11 mg. per 100 ml. Jacobsen [1933] obtained analogous results in experimental rickets. Heymann [1931] claims that the reduction of inorganic P of the serum in rickets is accompanied by increased ester P. The concentration of ester P in serum is so small, however (about 0·5 mg./100 ml.), that analytical results cannot be very reliable.

It remains to be decided whether the abnormal values observed in different P compounds are due purely to P starvation, or to a disturbance in the correct balance between the various P compounds due to the absence of the regulating action of vitamin D. Although a complete analysis of the different P compounds in the various tissues is at present impossible, by estimation of inorganic P, phosphagen, readily and difficultly hydrolysable esters, lipin- and protein-P, on a diet containing various levels of P and vitamin D, one should gain a fairly good impression of what takes place.

EXPERIMENTAL.

The experiments were carried out on rats. These animals were bred in the laboratory and fed on the same stock diet as their mothers from weaning until the commencement of the experiment, when they weighed about 50 g. They were then placed on one of the following experimental diets:

Steenbock-Black rachitogenic diet.

I.[1] 76 % maize meal.
20 % wheat gluten.
3 % $CaCO_3$.
1 % NaCl.
Ca : P = 4·7.

II. As I with vitamin D (50 I.U. calciferol daily, dissolved in arachis oil and administered by pipette).

P-rich diet.

III. 78 % maize meal.
20 % wheat gluten.
1 % $CaHPO_4$.
1 % NaCl.
Ca : P = 0·7.

IV. P-rich diet III with vitamin D (50 I.U. calciferol daily).

Overdosage of vitamin D.

V. Rats nos. 21, 22 and 23 were kept on the rachitic diet I until they developed typical rickets according to X-ray examination. They were then given massive doses of vitamin D over a very short period. No. 21 received 100,000 units 24 hours before killing; no. 22 received 50,000 units 48 hours and 24 hours before killing; rat no. 23 received 50,000 units on each of the 3 days before killing. These experiments were carried out in this way to ascertain whether a rapid interchange could be induced between any of the P compounds. Rachitic animals were used as the effect might be expected to be more clearly demonstrated in these. The results, as will appear later, were mostly negative, and in consequence the same experiments were repeated on two normal rats on a P-poor diet in order to have less interference by P absorption from the gut. These two rats were treated in exactly the same way as rat no. 23, thus receiving in all 150,000 units in 3 days.

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[1] Also called groups I, II, etc.

VI. As diet I but with addition of about 100,000 units vitamin D distributed over 10–14 days.

Condition of bones, etc. in various groups.

All the rats kept on diet I developed rickets according to X-ray observations in about 2 weeks. After killing the tibiae were cut by a longitudinal antero-posterior section. The bones were not well calcified, the proximal epiphyseal cartilage being about 1 mm. broad with irregular borders. The rats on diet II—normal according to X-ray—had epiphyseal cartilage about 0·5 mm. broad in upper tibiae with very regular sharp borders. The rats on diet III were all normal, the bones being better calcified than in group II and the epiphyseal cartilage only about 0·25 mm. broad.

The rats on diet IV were all normal, and had the most intensely calcified bones, with epiphyseal cartilage of the same shape and size as in group III.

Of the rats in group V the three rachitic animals had fresh deposits of bone salts in a broad epiphyseal cartilage; rat no. 23 however had very soft bones, indicating that, together with the deposit in the hypertrophic cartilage, a dissolution of already formed bone had taken place such as is well known in severe hypervitaminosis-D. In rats nos. 24 and 25 hardly any epiphyseal cartilage was recognizable, owing to fresh deposits of calcium salts; the bones, however, were not so fragile as in rat no. 23.

The rats on diet VI, also, had hardly any recognizable epiphyseal cartilage, but the sub-epiphyseal region seemed to be very sparingly calcified. Two rats in this group died; both had calculi in the pelvis of the kidneys. Thus the whole picture was that of typical hypervitaminosis-D. In Table III the body weight and average daily increase in body weight are given. These diets, well balanced with regard to Ca and P, seem to promote quicker increases in body weight than the P-poor Ca-rich diet. The average daily increase for the animals on diet V is given only until the commencement of overdosage, as this resulted in a loss in weight amounting to 5 g.

The rats were killed by decapitation and bled. During this period there occurred clonic convulsions, usually lasting 10–20 sec.; occasionally only a few spasms were seen.

Estimation of acid-soluble P.

As quickly as possible, samples of muscles (always the same muscles), liver and kidneys were weighed out. For extraction and analysis of the fractions of acid-soluble P in muscles the procedure of Eggleton & Eggleton [1929] was used with slight modifications in addition to those introduced by Mügge [1933] and King [1932]. The acid-soluble P of liver and kidney (one side for this purpose, the other for dry weight estimation), was extracted in the same way as for muscle, and inorganic, pyrophosphate and total P estimated in the usual way.

Instead of calculating with a presumed water content of the tissue as Eggleton did, the same dilution calculation as for blood was used.

The acid extracts of kidney and liver were neutralized with sodium hydroxide to prevent a possible liberation of inorganic phosphate from the esters as described by De Toni [1929] for blood filtrates.

Estimation of lipin- and protein-P.

As soon as possible—always within 30 min.—the precipitates were washed twice with about 15 ml. water to each g. of tissue, centrifuged and the supernatant fluid filtered through the same filter paper as had been used for obtaining the trichloroacetic acid filtrate. The precipitates were then transferred quantitatively to 100 ml. volumetric flasks with the aid of repeated washings with 96 % alcohol until a volume about 75 ml. was reached. They were then boiled 5–10 min. in a water-bath, cooled and made up to volume. The samples for lipin-P determination were obtained partly by filtration under watch glass and partly by centrifuging in tubes tightly covered with tinfoil. 15 ml. each of muscle and kidney filtrate and 5 ml. of liver filtrate were ashed with 1·5 ml. 60 % perchloric acid, and the P analysis carried out as previously described for the ashed samples. The proteins were centrifuged from the alcoholic lipin extract, washed once with about 15 ml. of alcohol and thereupon quantitatively transferred back to the 100 ml. volumetric flask by several washings with water. 3–4 N NaOH was then added giving a final concentration of about 0·10 N. The flasks were placed in a beaker of boiling water and boiled gently until all proteins were dissolved, cooled and made up to volume. Aliquot parts were then taken for P determinations, which were performed in the same way as the lipin-P estimation. Duplicate estimations were run on one sample of tissue in all the above-mentioned analyses. This was considered quite safe as duplicate experiments on two samples of muscle, liver and kidneys from the same animal always gave concordant results.

DISCUSSION.

The results obtained on all animals are given in Tables I, II and III, which give the results of the analysis on the muscles, liver and kidneys respectively. The body weight and average daily increase in weight are given in Table III.

Phosphagen. The results for muscle demonstrate that there is too great a variability in the phosphagen to allow any comparison at all. The variation is at least to some extent due to the changing intensity in the convulsions following decapitation; the highest values were always obtained when only a few spasms occurred. It was thought safer, however, to carry on with this way of collection of the sample. It always provided organs with approximately the same low content of blood, thus giving more homogeneous results. Samples taken from living narcotized animals are subject to considerable variation in blood content, as in the case of muscles and particularly the liver. According to Bauer *et al.* [1932] the liver acts as a blood reservoir for the right heart, storing or giving up blood under the influence of various stimuli. As the concentration in the blood of some of the P compounds under investigation is entirely different from the concentration in the tissue, a varying blood content might constitute a serious source of error. In the samples taken from these experimental animals only traces of blood were seen when the portal vein was cut; in muscles and kidneys hardly any blood was detectable.

Phosphagen and inorganic phosphate. We may next consider the sum of inorganic P and phosphagen-P as one group, since the result of a breakdown of phosphagen is a corresponding increase in inorganic phosphate. There is no phosphagen in liver and kidney, and we find that, in the same experimental

Table I. *Muscles.*

mg. P in 100 g. fresh tissue in

Rat no.	I Inorganic phosphate	II Phosphagen	I + II	Readily hydrolysable esters	Difficultly hydrolysable esters	Phospholipins	Proteins	% Water	Diet
1	36	21	57	33	58	55	44	75	Without D,
2	36	23	59	33	57	56	39	75	I
3	38	24	62	35	50	46	39	74	(Ca : P = 4·7)
4	33	29	62	31	60	47	40	76	
5	39	21	60	35	56	46	40	73	
6	46	22	68	35	54	50	37	76	With D,
7	50	16	66	38	64	51	41	74	II
8	54	13	67	32	60	53	44	73	(Ca : P = 4·7)
9	52	25	77	39	54	54	40	73	
10	42	39	81	37	51	50	41	73	
11	47	14	61	34	66	50	40	73	Without D,
12	37	28	65	37	61	49	39	71	III
13	51	20	71	31	56	52	43	78	(Ca : P = 0·7)
14	53	14	67	31	53	51	44	71	
15	54	16	70	35	65	46	44	74	
16	51	18	69	32	66	49	44	74	With D,
17	50	20	71	34	54	51	46	74	IV
18	54	16	70	36	66	48	43	74	(Ca : P = 0·7)
19	43	27	70	33	50	48	40	76	
20	56	19	75	32	53	51	43	74	
21	32	33	65	37	59	49	42	76	Hypervita-
22	38	35	73	41	51	49	40	71	minosis-D,
23	48	20	68	36	54	48	38	73	V
24	42	31	73	36	55	45	39	71	(Ca : P = 4·7)
25	43	37	80	37	57	44	40	73	
26	53	27	80	36	56	48	42	71	Hypervita-
27	32	40	72	32	53	47	41	75	minosis-D,
28	40	31	71	38	52	45	42	76	VI
29	38	31	69	30	59	46	42	74	(Ca : P = 4·7)

group, the values for inorganic P in liver and kidneys are much more regular than those for muscles. By comparing the inorganic phosphate plus phosphagen content of muscles, and the inorganic phosphate in liver and kidneys from animals on a rachitic diet (group I) with those from animals on the same diet but receiving a daily addition of vitamin D as protection against rickets (group II), we find a definite reduction in these compounds in tissue from the rachitic animals. Vitamin D therefore increases the inorganic P in tissues just as it has been shown to do in blood.

When the Ca content of the diet is reduced and the P content increased so that the diet is well balanced with regard to Ca and P, as in diet III, the animals do not develop rickets even when vitamin D is not added to the diet, and the inorganic phosphate in the tissue is kept at the same level as when vitamin D is added to a diet badly balanced with regard to Ca/P. The bones of these animals were even better calcified than the bones of the rats on the P-poorer diet with added vitamin D. Diet IV (addition of vitamin D to diet III) had no further effect on the inorganic P in the tissue than had diet III, but bone calcification was still better in this group. This is in agreement with the well-known fact that even with a good Ca/P balance in the diet, addition of vitamin D increases the retention of Ca and P. As there is no increase of these minerals in the soft tissue or in blood, it is clear that the increased deposition is confined to the bones.

Table II. *Liver.*

mg. P per 100 g. fresh tissue in

Rat no.	Inorganic phosphate	Readily hydrolysable esters	Difficultly hydrolysable esters	Phospholipins	Proteins	% Water	Diet
1	25	14	51	102	104	72	Without D, I (Ca : P=4·7)
2	26	12	54	101	106	71	
3	25	14	59	123	121	71	
4	23	16	55	107	106	72	
5	24	14	58	124	129	67	
6	35	14	54	146	126	72	With D, II (Ca : P=4·7)
7	29	18	62	160	132	67	
8	32	12	49	135	130	67	
9	32	13	54	149	136	71	
10	30	18	54	130	119	72	
11	28	13	61	130	120	67	Without D, III (Ca : P=0·7)
12	27	12	64	134	124	67	
13	30	12	61	127	127	69	
14	27	15	64	130	124	69	
15	29	13	60	138	128	69	
16	27	16	61	137	127	70	With D, IV (Ca : P=0·7)
17	29	12	63	126	115	69	
18	28	11	61	126	120	70	
19	35	10	59	157	140	71	
20	27	13	50	120	114	70	
21	29	15	53	130	130	67	Hypervitaminosis-D, V (Ca : P=4·7)
22	29	14	52	118	117	71	
23	28	13	61	121	116	70	
24	29	14	57	122	128	70	
25	30	13	57	137	128	70	
26	30	13	56	130	136	71	Hypervitaminosis-D, VI (Ca : P=4·7)
27	29	13	60	115	115	67	
28	26	13	55	119	110	68	
29	26	15	66	116	114	69	

The effects of excessive amounts of vitamin D on the inorganic P and phosphagen. Vitamin D was administered in excess in two ways as previously described. In the first series of experiments rats received massive doses for 1–3 days. As several authors have suggested that vitamin D might act by inducing a sudden liberation of inorganic P from organic compounds, this should be best demonstrated in experiments of short duration by administering very large doses of the vitamin to animals on a P-poor diet, under which conditions the absorption of P should influence the results to a small extent only. This experiment was first carried out on three rachitic animals; the only noteworthy result was an increase in the inorganic phosphate up to the level found in normal rats. Similarly this was the only effect obtained on two animals kept before the experiment on diet II (the same diet with normal amount of vitamin D).

Secondly, experiments were carried out on rats kept on a rachitic diet but given a more moderate overdosage of vitamin D over about 2 weeks. No effects were obtained on the liver and muscles beyond those induced by small additions of vitamin D to this diet or by balancing the same diet correctly with regard to Ca and P with no vitamin D added. An immense increase occurred in the kidneys; this was due to the deposition of calcium phosphate both in the tubule and in the pelvis of the kidneys, as is well known from the experiments of other authors, e.g. Harris & Moore [1929].

Table III. *Kidneys.*

mg. P per 100 g. fresh tissue in

Rat no.	Body weight g.	Average daily increase in wt. g.	Inorganic phosphate	Difficultly hydrolysable esters	Phospholipins	Proteins	% Water	Diet
1	62	1·0	30	51	100	89	79	Without D,
2	71	0·9	27	50	102	81	78	I
3	75	1·0	28	66	102	77	79	(Ca : P = 4·7)
4	68	1·0	30	55	102	77	79	
5	78	1·1	30	53	91	70	79	
6	68	1·2	36	62	111	92	77	With D,
7	57	0·8	33	57	107	87	78	II
8	57	0·9	37	46	111	84	79	(Ca : P = 4·7)
9	71	1·0	35	60	104	87	80	
10	72	1·0	33	61	109	85	76	
11	77	1·7	37	69	103	84	77	Without D,
12	80	2·0	35	52	115	91	79	III
13	78	1·5	38	62	108	82	78	(Ca : P = 0·7)
14	82	1·7	38	61	104	85	78	
15	79	1·4	39	62	110	95	77	
16	74	2·0	37	61	111	87	78	With D,
17	76	1·6	37	58	106	86	76	IV
18	73	1·5	39	64	116	80	—	(Ca : P = 0·7)
19	78	1·5	39	65	117	85	79	
20	63	1·4	38	60	108	84	76	
21	68	0·8	29	57	105	86	78	Hypervitaminosis-D,
22	73	1·0	39	56	101	73	79	V
23	75	1·2	37	61	107	77	79	(Ca : P = 4·7)
24	73	0·9	47	55	104	80	79	
25	74	1·0	49	64	103	80	79	
26	52	0	82	66	106	87	79	Hypervitaminosis-D,
27	61	0	63	56	111	88	78	VI
28	65	0	73	52	97	85	80	(Ca : P = 4·7)
29	66	0	53	61	107	86	79	

Variations in the esterified forms of P. In the readily hydrolysable esters—adenosinetriphosphate—we find the same variation in all six groups of rats, without any striking difference between one group and another. Occasionally we find a low value for inorganic P with a high pyrophosphate value in the same organ and inversely (compare livers from animals 16 and 19). This might appear to be due to analytical error. The pyrophosphate-P is the difference between P estimations after 7 min. hydrolysis by boiling with acid and a direct estimation of inorganic P in the same sample. When such values as mentioned above occurred, new analyses were made to ensure the correctness of the estimation. The same analytical results were always obtained. We know, however, that adenosinetriphosphate is formed by phosphorylation of adenylic acid. This may explain why an inverse variability of inorganic and pyrophosphate-P occurs in some animals.

The ester-P hydrolysable with difficulty—adenylic acid and hexosephosphates—varied from about 50 to 60 mg./100 g. in all organs and in each group of animals. Slight differences between the various groups were observed, but it is rather doubtful if the variations are significant.

That ester-P content can be influenced by further restriction of the P supply to the body is however shown impressively by Kay & Skill in their experiments with beryllium rickets. In the animals where very severe rickets occurred there was a depression in ester-P (sum of readily and difficultly hydrolysable ester-P) from 80 to about 30 mg./100 g.

In the lipin-P of the muscles there is, in all groups, the same slight variability, without any difference from one group to another; the same can be said about the protein-P in the muscles. The variability in these P compounds in the kidney is about the same as in the muscles but an evident though slight reduction in the rachitic animals is apparent when the groups are compared. In the liver the variability of phospholipin- and protein-P is greater, but here also there is a definite reduction in the rachitic animals. Contrary to expectation slightly lower values for these compounds were given by diet IV as compared with diet II which demonstrates that the normal variability is not properly registered by using only five animals in each group. The conclusion must therefore be drawn with some care. It seems, however, justifiable to conclude that on a P-poor diet both the phospholipin- and protein-P in liver and kidneys will be decreased to some extent. Why this does not take place in the muscles cannot be decided from these experimental results. We know, however, that both the liver and kidneys are far more susceptible to pathological anatomical changes than muscles. This is observed both in acute infections and intoxications. When the nocuous agent disappears from the body a rapid recovery to a normal anatomical picture takes place. This may explain why rats given excessive doses of vitamin D for long periods had lower acid-insoluble P in the liver than the other animals, with the exception of the rachitic rats.

It has been suggested by earlier authors that vitamin D acts by a sudden liberation of inorganic P from the acid-insoluble P compounds in tissue. If such a conclusion were to be justified the results should have been low acid-insoluble P in normal animals with an increase in rachitic animals. The inverse results have been obtained.

With regard to the acid-soluble P it is noteworthy that in the present work a decrease in the inorganic fraction was observed, which is in contrast with the decrease in the ester fraction reported by Kay and other workers. This apparent discrepancy may be attributable to the fact that the variability in normal and rachitic animals, as determined not only by body weight and daily increase in body weight but also by the length of experiment and P retention, has not yet been established.

When we find, as in these experiments, a depression in the tissue-P only after deprivation of vitamin D, and that this depression is eliminated by addition of P to the diet, the conclusion that the action of vitamin D in P metabolism, at least in rats, is to increase the supply of P from the intestinal contents to the blood stream, and thence to the tissue, seems well justified. There appears to be no evidence to support the view that vitamin D acts by maintaining a balance between the different forms of P in the organism.

SUMMARY.

1. In rachitic rats compared with rats on the same diet but with addition of therapeutic amounts of vitamin D there was a slight but definite reduction in the sum of inorganic P+phosphagen in muscles and in inorganic P in liver and kidneys. A reduction was also seen both in lipin-P and protein-P in the liver and kidneys from rachitic rats, but not in muscles. No definite change was observed in readily or difficultly hydrolysable esters in muscles or liver or in the difficultly hydrolysable esters in kidneys.

2. When P was added to the rachitic diet and the Ca content reduced, the animals remained normal and gave the same results for all P compounds as did animals on the rachitic diet with added vitamin D.
3. Addition of vitamin D to this P-richer diet, although giving better calcified bones, had no influence upon the amount of the different P compounds in muscles, liver and kidneys.
4. Overdosage by massive doses of vitamin D for short periods to rachitic rats increased the inorganic P to the normal level in muscles, liver and kidneys. The same experiments on rats given a rachitogenic diet, but protected from rickets by therapeutic amounts of vitamin D, showed only a slight increase of inorganic P in the kidneys.
5. The same massive amounts of vitamin D administered over 2 weeks to rats on a rachitogenic diet resulted only in an increase of inorganic P in the kidneys up to double the normal content, presumably through the formation of deposits.

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CXCI. SEASONAL VARIATIONS IN *CHONDRUS CRISPUS.*

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(*Received 13 June 1936.*)

PREVIOUS study of certain chemical properties of *Chondrus crispus* indicated that seasonal variations might be significant in the understanding of its metabolism [Butler, 1935]. With a view to obtaining such knowledge the present work was undertaken, the intention being to follow the changes noticeable at monthly intervals during the year.

In order to eliminate ecological variations other than those which were seasonal, a series of monthly collections was made from the same location, situated on MacNab's Island in Halifax Harbour [MacFarlane & Bell, 1933]. Collections were commenced in January 1931, and continued at monthly intervals until September when it was impossible to obtain another sample from that location. It was decided to begin collections at the same location in September of a year when no *Chondrus* had been collected previous to that month.

It was impossible to collect in December of that year, but collections were obtained in September, October and November 1934, and in January 1935; a collection was also made in November 1935 and has been used by way of check. Reference to the figures, in all of which the two January levels are close, indicates that the hiatus in collecting has had no serious effect on the results.

Treatment of collections.

The *Chondrus* plants were picked from spots scattered over the whole area mentioned above (approximately 750 sq. ft.). As the plants are small, any one collection contained a sufficient number to eliminate individual variations. When brought into the laboratory they were spread on tables and allowed to dry at room temperature. The plants were then carefully picked to free them of all foreign matter, both plant and animal, and when thoroughly cleaned the air-dried plants were ground in a grain mill. Samples were dried in an electric oven at approximately 80° for 6 hours or more.

Preparation of polysaccharide extracts.

From each of the monthly collections described above the polysaccharide extract was prepared according to a standard method previously described [Butler, 1934]. These are the extracts referred to throughout this paper.

COMPARISON OF WHOLE PLANTS OF *CHONDRUS* AND THEIR EXTRACTS.

Chondrus. From January to April the plants are dark in colour, a reddish or greenish black; from May till August the colour becomes lighter; whilst from September to January the colour gradually becomes dark again.

Extracts. The extracts are light and fibrous in texture, and are always sufficiently brittle to make it possible to powder them by grinding in a mortar.

Such slight variations as were noticeable in the appearance of extracts seemed, in general, to follow the natural colour variations of the whole plants.

Ash determinations (Fig. 1). Duplicate ash determinations were made on the monthly collections of *Chondrus* and on the extracts prepared from them. Ashing was carried out in an electric muffle furnace at approximately 600°. The results of the ash determinations on about 1 g. of the extracts were very satisfactory, only in one case was there a difference greater than 0·6 % between duplicates. The results for ash of the whole plants were not so constant as in the case of the extracts. Quite large differences occurred between duplicates, and many determinations were repeated. The maximum difference for any of the duplicates accepted for Fig. 1 was 1·6 %.

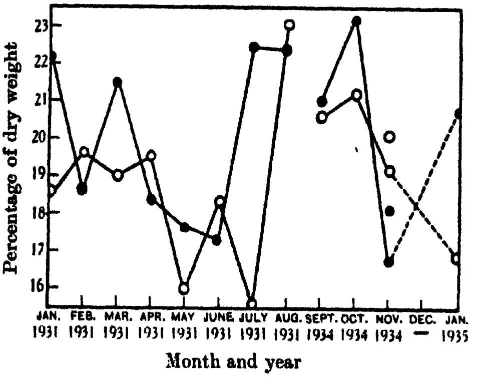

Fig. 1[1]. Ash in *Chondrus*.
• Whole plants. o Extracts.

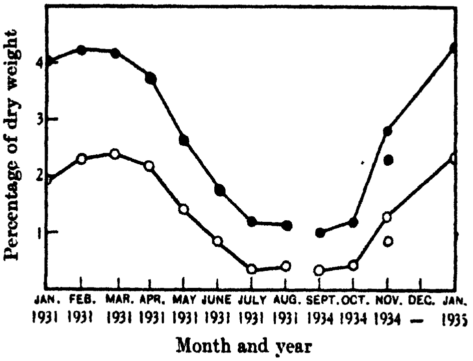

Fig. 2. Nitrogen in *Chondrus*.
• Whole plants. o Extracts.

In general the plants and extracts show the same series of fluctuations, the changes becoming apparent slightly later in the extract.

The percentage of ash is especially significant in the extracts, where only combined ash material is present. As has been pointed out previously [Butler, 1934] the standard extract is not a single chemical substance but is a mixture of closely related ethereal sulphates.

Any one, or all, of at least the following three types of sulphate esters may be present in the standard extracts:

Type I. $[R(OSO_2O)_2Ca]_n$.
Type II. $[(ROSO_2O)_2Ca]_n$.
Type III. $[HOSO_2O.R(OSO_2O)_2Ca]_n$.

Within any one of these types *R* may vary in complexity, in composition or in both; and *n* may vary, as well as the cation.

On ashing, types I and II break down in such a way as to retain in the ash as $CaSO_4$ one-half of the total sulphate; in type III, however, only one-third of the total sulphate will be retained in the ash.

It is suggested that in esters of these types a low complexity of carbohydrate will be associated with a high percentage of ash and from variations in the latter it might be possible to obtain some indication of the complexity of the carbohydrate radicals. The results obtained will be discussed later in relation to these considerations.

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[1] In each figure, the August and September points have not been joined because of the break in collecting at that time. Also, the November 1934 and January 1935 points have been connected with dotted lines because of the absence of the intermediate point. The extra point in each represents November 1935.

Nitrogen.

The percentages of nitrogen in the monthly collections of *Chondrus* and in the extracts from each were determined by the Kjeldahl method. The results are expressed graphically in Fig. 2.

It is readily seen that the percentage of nitrogen in the extracts depends directly on that present in the whole plants. This observation had been previously made on extracts prepared from samples collected at widely separated places, as well as at different seasons [Butler, 1935].

Carbohydrate in whole plants and extracts.

Since the amount of fatty material in *Chondrus* is negligible, the percentage of carbohydrate has been calculated as the difference between 100 % and the sum of the percentages of crude protein and ash.

Fig. 3 shows these results graphically, and it is evident that the general trend of variations is similar in plants and extracts.

From the foregoing data, the assumption that variations in the extracts are expressive of variations in the plants themselves seems justified. From this point, therefore, attention has been directed to a more detailed study of the changes taking place in the extracts, as being significant of those in *Chondrus* itself.

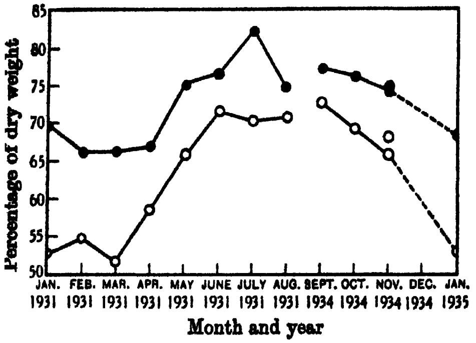

Fig. 3. Carbohydrate in *Chondrus* by difference. o Whole plants. • Extracts.

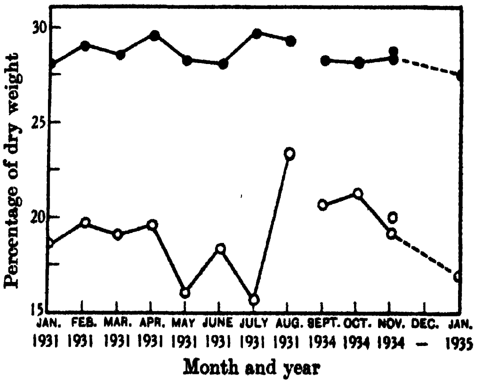

Fig. 4. Ash and sulphate in *Chondrus* extracts. • Total sulphate. o Ash.

Variations in the extracts.

Sulphate. The percentage of total sulphate in each extract was estimated, after hydrolysis in 5 % HCl, by precipitation with 10 % $BaCl_2$. A weighed sample (approximately 0·5 g.) of the extract was boiled for 5 hours under a reflux condenser in 250 ml. of 5 % HCl. The hydrolysed solution was then made up to volume again and 25 ml. aliquots were used for the sulphate determination. Aliquots of this same hydrolysate were used for estimating the percentage of reducing sugars present.

The total sulphate results, given in Table I, col. (1), are averages of closely agreeing duplicates.

In Fig. 4 the percentage of total sulphate in the extracts is compared with that of ash.

Table I. *Percentage of sulphate in extracts.*

Collected	(1) % SO_4 (after hydrolysis)	(2) % SO_4 calculated from ash	(3) % SO_4 lost in ashing	(4) % total SO_4 retained in ash
January 1931	27·97	9·72	18·25	34·76
February 1931	29·00	10·04	18·96	34·61
March 1931	28·53	10·07	18·46	35·28
April 1931	29·57	10·23	19·34	34·58
May 1931	28·13	9·42	18·71	33·50
June 1931	28·04	11·18	16·86	39·88
July 1931	29·70	9·55	20·15	32·14
August 1931	29·25	13·77	15·48	47·09
September 1934	28·21	12·06	16·15	42·75
October 1934	28·07	12·68	15·39	45·15
November 1934	28·31	10·87	17·44	38·42
January 1935	27·49	8·76	18·73	31·87
November 1935	28·56	11·69	16·87	40·95

They are seen to correspond closely until May, after which, until November, when a falling off occurs in both, there appears to be no correlation. The sulphate is a better indication of actual combined inorganic material than ash, because of the uncontrollable factors which may enter into the estimation of the latter. Sulphate was also estimated in the ash from each sample, and the amount present calculated as % SO_4 in the original extract. These figures, also, are given in Table I, col. (2), and, along with them, col. (4), the percentage of total SO_4 retained in the ash.

Carbohydrate as reducing sugar. The reducing sugars present in the hydrolysate described under "Sulphate" were determined in 2 ml. aliquots by the Hanes modification of the Hagedorn-Jensen technique [Hanes, 1929]. The results, expressed as percentage carbohydrate calculated as glucose, are given in Table II, col. (2), which includes also the percentages of carbohydrate obtained by difference, col. (1), corrected for the sulphate lost on ashing.

Table II. *Percentage of carbohydrate in extracts.*

Collected	(1) Carbohydrate by difference (corrected)*	(2) Carbohydrate found calculated as glucose
January 1931	51·31	53·10
February 1931	47·05	50·83
March 1931	47·55	50·88
April 1931	47·39	49·75
May 1931	56·56	52·95
June 1931	59·68	53·84
July 1931	62·31	54·90
August 1931	59·12	53·71
September 1934	61·27	56·64
October 1934	60·94	55·34
November 1934	56·93	54·67
January 1935	49·75	53·10
November 1935	58·04	54·17

* The correction was made by deducting the percentage of sulphate actually lost (see Table I, col. (3)) as well as the ash and protein.

The figures obtained by the two methods may be seen to agree fairly well during the low level of the early part of the year. In May, carbohydrate, calculated by difference, shows an increase; it may be that something is lost in ashing and is being calculated as carbohydrate by difference, though it has no reducing power. The same sequence of changes is evident in both, even though the level is different, which suggests that relative values are correct although the absolute ones may not be.

In Fig. 5 the variations in carbohydrate (calculated as glucose) are shown graphically, in relation to protein and sulphate.

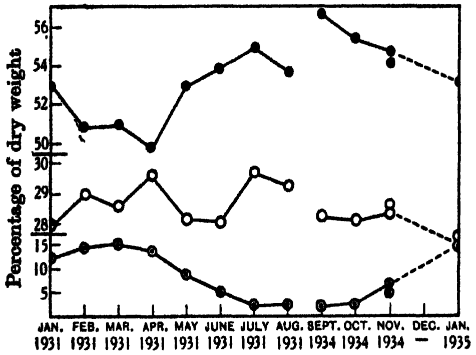

Fig. 5.[1] Carbohydrate, total sulphate and protein in extracts of *Chondrus*. ● Carbohydrate. ○ Total sulphate. ⊙ Protein.

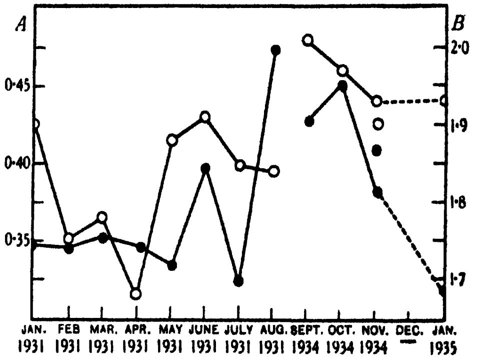

Fig. 6. Sulphate ratios in *Chondrus* extracts. $A, \dfrac{\text{Sulphate in ash}}{\text{Total sulphate}}$ ●. $B, \dfrac{\text{Carbohydrate}}{\text{Total sulphate}}$ ○.

For the sake of clarity in the discussion which is to follow, two ratios have been included in Fig. 6; *A*, % SO_4 in ash/% total SO_4, and *B*, % carbohydrate (calculated as glucose)/% total SO_4.

DISCUSSION.

As suggested in the section on ash determinations, variations in the percentage of ash in the extracts may be some indication of the complexity of the carbohydrate. By reference to Figs. 4 and 5 it may be observed that the experimental results only partially coincide with this assumption. During the early part of the year the percentage of ash is fairly high and that of carbohydrate low. Also, the period of low ash level, May to July, corresponds with the high carbohydrate one. From about October, however, percentages of both fall off together, suggesting the intervention of other factors.

In considering the relation between ash and carbohydrate it should be remembered that for a similar carbohydrate complexity in all three esters, each will leave a different percentage of ash.

Fig. 6, showing graphically the ratio of sulphate in ash to total sulphate, may be considered in interpreting the results, especially with reference to the type of ester predominating. It will be observed that from the beginning of the year until May, this ratio is such as to indicate a predominance of the acid salt (type III), in which it is 0·333. The ratio rises to that required by esters of type I or II (0·500) about the time (August) when the ash rises. From October, this ratio and the percentage of ash both fall off, which, taken together, might indicate a return to the acid salt.

How do carbohydrate variations fit in with these ash and sulphate variations? The low carbohydrate level from January to April may account for the rise in sulphate percentage which accompanies it. In April when carbohydrate begins to rise, the percentage of sulphate drops, but from June to October they increase and then decrease together. During the time, then, when the previously considered evidence seemed to point to predominance of the acid salt, the percentage of carbohydrate is low. At the same time as it begins to increase, there is, according to the ash-SO_4/total SO_4 ratio, a change to the normal type of salt. Simultaneously with the decrease in percentage carbohydrate (i.e. from October) there is, according to the sulphate ratio, a gradual return to the acid type of salt.

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[1] The scale of percentages in Fig. 5 is not continuous. In order to include the three graphs in the same figure, two breaks have been made.

Fig. 6 shows that the ratio is low until April, when it begins to increase along with carbohydrate; from September, it begins to fall, again along with carbohydrate. While the percentages of both sulphate and carbohydrate are increasing there is a drop in the ratio, so that even though the carbohydrate is increasing, it is not doing so in proportion to the sulphate. This drop of ratio in July, therefore, would seem to signify a greater increase of sulphate, such as would be necessary for a change to the normal salts. In July, when the lowest ash-SO_4/total SO_4 ratio point occurs, a high percentage of carbohydrate is reached; but the percentage of sulphate is also high at this time. The steep rise in the ash-SO_4/total SO_4 ratio, which follows this low point, would seem to indicate a more or less sudden change from type III salts to type I or II. Further evidence in support of this change in type is provided by the high level of carbohydrate/SO_4 ratio from September to January. From these considerations then, it appears that the normal salts predominate during the summer months, when the ash-SO_4/total SO_4 and carbohydrate/SO_4 ratios (and ash) are high.

A possible explanation might be that active assimilation beginning about April is responsible for the increase in the carbohydrate/SO_4 ratio. From September, when it may be assumed the reserve of carbohydrate material is highest, there is a gradual decrease of both carbohydrate and sulphate and, according to the sulphate ratio, a simultaneous reversion to the acid salt, as the supply of normal esters becomes finally exhausted in January. From then on until about April there is a decided drop in the carbohydrate/SO_4 ratio. The evidence of the sulphate ratio points to a predominance of the type III ester during this time.

Comparison of the curves for total sulphate and for carbohydrate shows them to be more or less reciprocal until June, after which they fluctuate together until January. During this latter period a high level in their ratio occurs, so that it seems justifiable to assume the carbohydrate complexity to be high during that period, even after the drop of the ash-SO_4/total SO_4 ratio indicates a return to the type III ester.

From January until about April, i.e. during the time of most active catabolism, the carbohydrate/SO_4 ratio drops, possibly as the carbohydrate complexity decreases, whilst during the active anabolic phase, April to September, this ratio increases.

It may be that the acid type of salt is the fundamental basis of the extract, or of the carbohydrate economy of the plant, and is always present. As a hemicellulose it can be used in metabolism if required, but the normal esters are the surplus or storage forms which are first used. When the supply of these is exhausted, the ordinary acid ester is metabolized by a reduction in its carbohydrate complexity.

SUMMARY.

A series of monthly collections of *Chondrus crispus*, made from the same location over a year period, is compared with the polysaccharide complex extracted from them in regard to ash, nitrogen and carbohydrate content.

Similar variations in ash content occur in both, though they are apparent later in the extracts than in the whole plants.

The nitrogen present in the extracts is found to depend directly on the amount present in the *Chondrus* plants.

The percentage of carbohydrate is low until March or April when a rise begins and a high level is reached extending from May to October.

The relationships of the sulphate retained in the ash and the total sulphate of the extracts indicate predominance of an acid type of ester from January to May. From May till October esters of the normal type predominate. After October, until January there appears to be a gradual return to the acid type of salt.

The relations of carbohydrate to total sulphate also indicate that the acid salt predominates from January to about May.

The hypothesis is suggested that the supply of the carbohydrate complex synthesized during the summer months is largely of the normal ester types, and that after the supply of these has been used up, the acid type of ester alone remains to be metabolized.

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CXCII. CORTICO-ADRENAL INSUFFICIENCY AND POTASSIUM METABOLISM.

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(*Received 11 June 1936.*)

I. DETERMINATION OF POTASSIUM IN SMALL QUANTITIES OF BLOOD AND TISSUES.

A RISE in blood potassium following adrenalectomy was first reported by Baumann & Kurland [1926], who found an average value of 28 mg./100 ml. in a series of 11 cats. Hastings & Compere [1931] found that serum potassium rose in dogs from an initial average concentration of 3 *mM* to one of up to 20 *mM*; these authors suggested that very similar values are encountered in potassium poisoning. Their paper does not, however, give any experimental details, such as the method for the determination of potassium or at which stage of adrenal insufficiency the samples were taken; moreover, the concentrations for normal dogs are distinctly lower than those reported by other authors. Kerr [1926], finds 4·7–5·6 *mM*, and the insufficiency values are higher than those reported by different workers. Zwemer & Sullivan [1934] reported an average value of 34·7 mg./100 ml. (22 determinations) with higher values in later insufficiency. In the human subject, Marañon *et al.* [1934] find maximum pre-mortal values of 49·5 mg./100 ml. with a mean value of 31·1 mg./100 ml. in Addisonians. Urechia *et al.* [1935] state that serum K of cats may rise to as much as 250 mg./100 ml. 10 min. after adrenalectomy (7 cats, survival time 15–64 hours). The very short survival time of their animals suggests that death supervened rather from operative damage than from adrenal insufficiency, whilst the high values reported are indicative of some methodical error, since we have found that the extreme values, obtained at death from cats poisoned with potassium, do not exceed 70 mg./100 ml. (unpublished work). Loeb *et al.* [1933] ascribed the high potassium values in dogs to haemolysis, with liberation of K from the erythrocytes; since, however, the K content of dog corpuscles is not significantly higher than that of the plasma [Kerr, 1926] it should rather be considered that a high plasma K is also a symptom of adrenal insufficiency in dogs.

Whilst high blood potassium has been found by most investigators in cortico-adrenal insufficiency, the actual levels found vary widely, and no systematic attempt has been made to correlate the symptoms with the blood potassium level. Further, the results are open to objection on several counts. Thus, Kerr [1926] has shown that the blood potassium of dogs may rise from 6·5 to 15 *mM* as a result of repeated haemorrhage. The amounts of blood taken for sampling by certain of the workers cited above were such as might be expected to lead to a similar result, as well as definitely to shorten the survival period.

In the case of human blood, haemolysis of even a small percentage of the erythrocytes might lead to a very high value for potassium. Whilst it is our opinion that Marañon's results are not due to this cause but represent the actual conditions in Addison's disease, yet it is felt that a reinvestigation of blood potassium values in human adrenal insufficiency is desirable.

In view of the above, the present reinvestigation of the behaviour of blood potassium during the course of experimental adrenal insufficiency in cats was undertaken. The research involved the elaboration of a method which would allow of the analysis of small quantities of peripheral blood; it was then possible to take daily samples without thereby significantly affecting the course of the syndrome.

Since the method involved taking whole blood, the most suitable experimental animals were cats, in which Abderhalden [1898] has shown that potassium is equally distributed between plasma and cells.

EXPERIMENTAL.

Known amounts of potassium (0·02–0·16 mg.) were determined by a colorimetric method based on that of Breh & Gaebler [1930] taking a standard containing 0·1 mg. K. The results are represented by curve 1, Fig. 1; the curve, being rectilinear, has the general equation $y=ax+b$, where x and y are respectively mg. K found and taken; by substituting different values of x and y, a is found to be 0·9, and b, 0·01; the final form of the equation is then

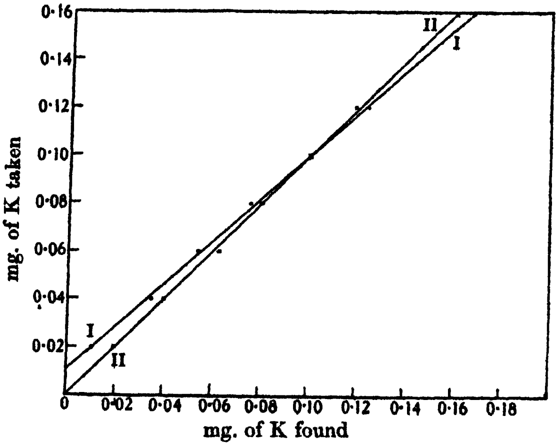

Fig. 1. All points represent the mean of 4 determinations.

$$y=0{\cdot}9x+0{\cdot}01.$$

The same equation may be derived on the assumption that, under the given standard conditions, a constant loss of the precipitate takes place, irrespective of the actual amount present; if this be represented by k, the result of the colorimetric reading may be represented by

$$\frac{S-k}{y-k}=\frac{S}{x},$$

where S represents mg. of K in the standard, and y and x have the same significance as before. Substituting 0·1 for S, and different values for x and y, it will be found that $k=0{\cdot}01$, and substituting this value in the second equation, and simplifying, it again assumes the final form $y=0{\cdot}9x+0{\cdot}01$.

It can be concluded that accurate values will be given by methods of the type in question only when the sample under analysis has the same K content as the standard, and that increasingly divergent values will be obtained the greater the difference in K content. Further, these results are adequately explained on the assumption that a constant loss,[1] equal to 0·01 mg. K, takes place under the given conditions. The results obtained should therefore be corrected accordingly; a simpler procedure is to add 0·01 mg. K to all the tubes, and this was adopted in the method described below. Curve II, Fig. 1, shows the results obtained for the determination of 0·02–0·18 mg. K; it will be seen that values little different from theoretical are obtained over the whole range studied.

Methods.

Special reagents.

A. Standard K_2SO_4 solution containing 2 mg. K per 100 ml.

B. Precipitating reagent: 210 ml. of a solution of 120 g. of K-free $NaNO_2$ in 180 ml. of water are added to a solution of 25 g. of $Co(NO_3)_2$ in 50 ml. of water and 12·5 ml. of glacial acetic acid, and air is bubbled through the solution for 1–2 hours to complete elimination of NO_2. The solution should be kept at 0° and re-aerated weekly; after a month it is advisable to reject it. When taken for use, the clear liquid is decanted off, a tenth of the volume of 40% $AgNO_3$ is added, and the solution is shaken and filtered.

C. 0·5% sulphanilic acid in 30% CH_3COOH.

D. 0·5% α-naphthylamine in 30% CH_3COOH.

In the actual determination of K in blood, duplicate 0·2 ml. samples of whole blood were taken in oxalated blood-pipettes from an ear vein, rejecting the first drop appearing after making the incision. They were immediately diluted in 5·9 ml. of water in a 15 ml. conical pyrex centrifuge-tube. 0·4 ml. each of 10% sodium tungstate and 2/3 N H_2SO_4 were added, the mixture was shaken, 0·1 ml. of 5% $AgNO_3$ was added, the tubes were again shaken and centrifuged (3000–4000 r.p.m.; 15 min.). 5 ml. of each centrifugate were transferred to 15 ml. graduated centrifuge-tubes, the tubes were warmed to 60–70°, and 2 ml. each of reagent *B* were added, as well as to two tubes containing 5 ml. of standard K_2SO_4 solution (solution *A*); 0·5 ml. of solution *A* had previously been added to all tubes. Two hours later the tubes were centrifuged, the supernatant fluid was siphoned off (leaving 0·2 ml.), and the precipitate of K argenticobaltinitrite was washed 3 times on the centrifuge with three 7 ml. portions of water. 5 ml. of 0·2 N NaOH were then added to the precipitate in each tube, the suspension was boiled to break down the complex, cooled, diluted to exactly 6 ml. in each tube and centrifuged. 1 ml. of each of the centrifugates was transferred to 50-ml. volumetric flasks containing 15 ml. of 10% acetic acid. 2 ml. of sulphanilic acid and 1 ml. of α-napthylamine solutions were added to the flasks, which were then filled to the mark with 10% acetic acid and allowed to stand for 10 min., when the colours were compared in a Duboscq type colorimeter, using a blue filter. The potassium content of the blood is given by

$$\text{mg. K/100 ml.} = \frac{S \times K_S \times 100}{R \times V},$$

where S is the level at which the standard is set (usually 20), K_S is the amount of K actually taken for the reading (0·1/6 mg.), R is the reading of the unknown, and V is the volume of the blood corresponding with the amount of NaOH solution finally taken; should this be 2 ml., $V = 0{\cdot}2 \times 5/7 \times 2/6$. Hence mg. K/100 ml. is in the given case equal to $70S/2R$.

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[1] This loss probably represents the difference between loss due to solubility of K argenticobaltinitrite, and a gain due to presence of minute amounts of K in the reagents. For these reasons it will be obvious that *k* should be determined empirically in each laboratory, and that it should be redetermined should any of the reagents be procured from a new source.

If the sample under analysis contains $< 0{\cdot}05$ mg. of K, it will be desirable to take 2 ml. or more of the final centrifugate, but if its K content is $> 0{\cdot}2$ mg., it will not be possible to compare the colour with that of the standard, owing to formation of a flocculent precipitate; in such cases, the amount of final centrifugate taken should be reduced to 0·5 ml.

Presence of Ag in the blood centrifugate may lead to considerable error in the determination, as under the given conditions sparingly soluble, colourless needles of $AgNO_2$ tend to separate out together with the precipitate of K argenticobaltinitrite. These crystals will, if present in small amount only, be eliminated during the process of washing; otherwise part of them remains and leads to the obtaining of fictitiously high values for K. In normal blood, the amount specified (0·1 ml. of 5 % $AgNO_3$) is slightly less than that required for complete precipitation of Cl^-; in the hypochloraemia sometimes encountered in advanced adrenal insufficiency it is probable that some small excess of $AgNO_3$ remains, but this has not been observed to interfere. If, however, the laboratory temperature falls below 15° during precipitation, the crystals will often separate in considerable quantity; this is avoided by maintaining the temperature at about 20°.

A further source of error lies in the fact that NH_4^+ is precipitated together with K; with the small amounts of ammonia present in freshly drawn blood this will not significantly affect the results, but where the sample has been allowed to stand at room temperature for any length of time considerable error may result. It is therefore advisable to deproteinize as soon as possible after collection of the sample and to keep the centrifugates in the ice-box should precipitation of K not be effected immediately.

With the given amount of blood, deproteinization was found to be incomplete when less than the specified amount of the appropriate reagents was taken.

In late adrenal insufficiency the peripheral circulation is highly inadequate, and at this stage it is often necessary to take blood from the heart. In such cases, and for plasma or serum, the following procedure has been applied; 0·5 ml. of the sample is diluted to 7·7 ml., 1·0 ml. each of tungstate and sulphuric acid solutions and 0·3 ml. of 5 % $AgNO_3$ are added, and 5 ml. of the centrifugate are taken for the determination.

Application to determination of potassium in muscle.

0·1–0·3 g. of skeletal or cardiac muscle is weighed in a tared, graduated pyrex centrifuge-tube, and subjected to wet incineration, according to the method of Leulier & Bernard [1934] (heating on a gauze with 0·5 ml. of a mixture of 35 ml. of $HClO_4$ and 65 ml. of H_2SO_4); the heating is continued to almost complete elimination of HCl, and the colourless solution is then diluted with 2 ml. of water and made neutral by adding a small excess of Li_2CO_3. The excess of the latter is dissolved by adding a few drops of glacial CH_3COOH, 2 ml. of 20 % $NaNO_2$ and 0·5 ml. of glacial CH_3COOH are added, and the tube is heated at 100° for 30 min. 0·1–0·3 ml. of 5 % $AgNO_3$ is then added, the solution diluted to 10 ml. and 1 ml. transferred to a second tube containing 4 ml. of water. K is then determined by the method described above for blood, when, if equal volumes of the final centrifugates are taken, mg. K/100 ml. is given by $100\,S/WR$, where W is the weight of tissue taken.

The above procedure is applicable to any other tissue. Where only very small amounts of tissue are available, the steps of dilution and transference of an aliquot part of the solution could be eliminated; the amount of tissue taken for incineration should not be less than 5–10 mg., containing 0·02–0·04 mg. K.

Speed and accuracy of the method.

The time required to perform 7 duplicate determinations of blood potassium has been found not to exceed 7–8 hours, if the operations of siphoning off the liquid and washing the precipitate in 8 tubes are performed while the remaining tubes are centrifuged.

No very high degree of accuracy is claimed, as the results obtained in parallel determinations often vary by ± 5 % from the mean value.

II. BLOOD POTASSIUM OF NORMAL AND ADRENALECTOMIZED CATS.

A. *Normal cats.*

Abderhalden [1898] gives the K content of normal cat blood as being 21·6 mg. per 100 g., in serum, whole blood and cells. Baumann & Kurland [1926] report an average value of 19·0 mg. per 100 ml. (varying from 15·9 to 24·4) of plasma, and 24·1 (19·7–28·8) mg. per 100 ml. for whole blood. D'Silva [1934] finds that the K content of serum varies from 16 to 22 mg./100 ml. and the values reported by Zwemer and Sullivan[1] for normal cat serum (15–22 mg./100 ml.) likewise fall within very similar limits.

In a series of 19 normal cats (males, 3·5–4 kg. in weight) a mean value of 19·3 (14–23·1) mg./100 ml. of K was found for plasma, using the methods described in Part I. The same animals had values of 20·2 (16·2–26·1) mg./100 ml. for serum, and 21·4 (16·0–26·1) mg./100 ml. for whole blood. These values are substantially identical with those of previous workers, and confirm the equal distribution of K in the cellular and non-cellular constituents of the blood of cats.

B. *Adrenalectomized cats.*

The adrenal glands were removed under ether anaesthesia, and under conditions of strict asepsis, either by a two-stage operation (dorso-lumbar incision), allowing 1–3 weeks to elapse between the first and the second operations, or by a single operation (mid-abdominal incision), applying a new technique described elsewhere. The survival periods were similar with either procedure so that the single operation was accordingly performed in most cases. The cats recovered rapidly from the operation, as far as could be judged from their appetite and activity; the animals were allowed tinned salmon and milk *ad lib.*, and in general no marked anorexia was evident earlier than the fifth day after the operation. Care was taken to maintain the laboratory temperature as far as possible constant (about 20°), in view of the known sensitivity of adrenalectomized animals to temperature fluctuations. The average survival time was 10 days, and blood was taken at intervals after the operation.

The results are given in Table I, from which it appears that the average blood K rises during the first 3 days following adrenalectomy, to a maximum value of

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[1] The figures in this paper are erroneously given as mg. K/100 ml.; they should be mg. KCl/100 ml.

Table I. *Blood potassium in adrenal insufficiency.*

Each value is the mean (mg./100 ml.) of two concordant determinations, on whole blood, plasma or serum.

Cat no.	Days following bilateral adrenalectomy														Survival in days
	0	1	2	3	4	5	6	7	8	9	10	11	12	>12	
3623	22·2	27·3	—	33·3	29·4	—	—	—	—	—	—	—	—	—	5
3504	17·0	—	—	—	20·5	21·7	27·5	—	—	—	—	—	—	—	6
3509	21·0	—	—	—	—	—	27·6	—	—	—	—	—	—	—	6
3503	20·0	—	—	—	—	—	22·4	—	—	—	—	—	—	—	7
3605	20·0	—	—	41·0	—	33·5	—	—	—	—	—	—	—	—	7
3601	—	—	—	—	24·6	—	44·4	—	44·4	—	—	—	—	—	8
3506	—	—	—	—	—	—	23·2	—	—	18·3	—	—	—	—	9
3512	21·3	—	—	—	—	—	31·1	—	—	31·2	32·0	—	—	—	10
3508	20·0	—	—	—	—	—	—	—	22·5	—	24·2	—	—	—	11
3627	22·1	25·2	28·5	37·8	35·0	22·3	20·5	21·0	11·8	11·1	17·1	17·5	22·7	45·7	13
3624	24·2	22·2	—	31·2	39·3	27·1	29·6	31·1	32·2	25·7	23·5	24·8	22·8	26·3 25·2 22·1	15
3510	20·0	—	—	—	30·6	—	—	21·5	33·3	—	—	—	—	27·5	16
3602	—	—	—	42·5	—	44·2	—	—	—	39·0	—	31·6	—	35·0 35·1 31·3	17
3603	17·5	—	—	—	—	36·3	—	—	32·2	—	34·2	—	37·5	41·5 46·1 31·0	31*
3611	—	—	21·4	—	—	26·9	30·2	—	—	—	—	—	—	—	†
3621	—	—	25·3	32·8	—	—	—	—	—	—	—	—	—	—	†
3628	16·0	—	28·0	—	—	—	—	—	—	—	—	—	—	—	†
3629	—	—	30·2	—	—	—	—	—	—	—	—	—	—	—	†
Average	20·1	24·9	26·7	36·4	30·0	30·3	27·9	24·5	29·4	25·1	26·2	24·6	27·7	34·2	—
No. of determinations	12	3	5	6	6	7	9	3	6	5	5	3	3	11	—

* Used for other experiments. † Probably had accessory tissue.

36·4 mg./100 ml., thereafter fluctuating. Considerable variations are found during the course of the syndrome, depending on the stage of insufficiency and the individual behaviour of the animals. Blood K substantially identical with normal may be encountered and, unless daily samples are taken, particularly during the first 3–5 days after adrenalectomy, the higher values may be missed. Cats 3624 and 3627 drank considerable amounts of water after the fifth day following adrenalectomy, and this, together with their restricted food intake, might explain the low values found subsequently.

The average value for the entire group agrees most closely with that of Baumann & Kurland [1926], but is also little different from that given by Zwemer & Sullivan. No values even approaching the maxima recorded by Hastings and Compere or by Urechia *et al.* have been found.

The values given in Table I represent results obtained for whole blood, plasma or serum. Although satisfactory evidence is available that in normal cats identical results are obtained for cells and plasma, yet the possibility remained that the rise in whole blood might apply only to the plasma, or to the cells. In

Table II. *Distribution of potassium between cells and plasma in adrenalectomized cats.*

Each value is the mean of two determinations.

Cat no.	Days after adrenalectomy	mg. K/100 ml. in		
		Cells	Plasma	Whole blood
3504	6	21·3	21·7	17·7
3504	7	—	27·5	30·5
3506	10	—	18·3	15·6
3510	16	36·2	37·5	37·8

order to exclude this objection, potassium was in some cases determined separately for whole blood, plasma and cells (Table II); it is evidence that the distribution is uniform in the cases studied.

III. POTASSIUM CONTENT OF SKELETAL AND CARDIAC MUSCLE IN CORTICO-ADRENAL INSUFFICIENCY.

Samples of muscle were taken from the adductor femoris and from the heart of animals killed when in the terminal stage of insufficiency, or immediately after death, and K was determined by the method described above.

Table III. *Potassium content of muscle.*

Each value is the mean of two determinations.

Cat no.	Days after adrenalectomy	Dry content of muscle	mg. K/100 g. in Wet muscle	mg. K/100 g. in Dry muscle
		Skeletal muscle.		
Normal	--	28·2	501·6	1778
—	-	27·2	459·2	1689
		Adrenalectomized.		
3609	8	22·4	437·7	1954
3615	9	20·6	408·1	1981
3627	12	21·1	382·0	1811
3624	15	20·2	375·4	1858
3602	17	22·8	431·5	1893
3603*	31	24·1	456·7	1895
	Average	21·9	415·2	1900
		Cardiac muscle.		
3615	9	21·9	305·8	1396
3627	12	22·0	309·0	1405
3624	15	20·5	211·2	1030
	Average	21·5	275·3	1277

* Presence of accessory cortical tissue probable.

The results given in Table III indicate that the K content of the fresh muscle is lower than in normal animals (the literature gives values of 450–500 mg./100 g.). At the same time, the water content is higher, so that the results calculated per 100 g. of dry muscle are actually higher than for normal cats.

Leulier *et al.* [1933] give the potassium content of normal cat myocardium as 365–368 mg./100 g. the values found in insufficiency will be seen to be significantly lower.

Discussion.

It may be concluded on the basis of the above evidence that, in cats, bilateral adrenalectomy is followed by a rise in blood K; this in most cases attains its maximum on the third or fourth day after the operation, thereafter falling to a more or less constant level of 30 mg./100 ml. average, which is also the level most often encountered at death. In individual cases normal and subnormal values may be encountered in the period following the initial rise.

The high K characteristic of cortico-adrenal insufficiency may be due to disturbances in the renal excretory function, to liberation from or lack of fixation in the tissues, or to both of these causes. Were the kidneys alone to be at fault, it might be expected that the rise in plasma K would be associated with a rise in tissue K; actually the results obtained for muscle indicate a subnormal value. On the other hand, there is no reason to believe that a normally functioning kidney could not deal adequately with the not very great amounts of K liberated from the tissues. It would hence appear to be most probable that, whilst both the causes mentioned may take effect simultaneously, the more important would be inability to eliminate K (exogenous or endogenous) entering the blood stream.

From this viewpoint, the variations in blood K following adrenalectomy may be interpreted in the following way. Anorexia does not appear until the third or fourth day after the operation; this coincides with the first high level. Up to this point, K of alimentary origin has been undergoing resorption and has tended to accumulate in the blood stream. Following this, the animals on an unvaried diet tend more and more to limit their food intake and to drink water; at the same time progressively lower values are found for blood K. The activity of the cats varies parallel with their appetite, and since muscular activity is associated with passage of K from the muscle cells to the plasma, this would function as a second factor, tending to introduce K into the blood stream during the first period of rising K and to withhold it during the succeeding stage. The final rise in blood K is ascribable to tissue breakdown, due to inanition, and to progressive aggravation of the kidney lesions.

It has been suggested by Hastings and Compere and by Zwemer and Sullivan that the higher values for blood K found by them are of the same order as those found in animals dying after intravenous administration of a lethal dose of KCl. Marañon *et al.* ascribed the symptoms of Addison's disease to defective regulation of water metabolism, due in turn to the high blood K, and Urechia *et al.* stated that the muscular debility of adrenal insufficiency might be due to K poisoning.

The most striking symptoms encountered in cortico-adrenal insufficiency are anorexia, muscular weakness, low blood sugar, low blood Na, high N.P.N., degenerative changes in the proximal renal tubules, vascular disturbances involving stagnation of the peripheral circulation and anhydraemia. At death, the heart is invariably found in diastole, often with considerable dilation. Experiments performed by the present authors on normal and adrenalectomized cats [Zwemer & Truszkowski, 1936] have shown that all the symptoms enumerated may be reproduced in normal animals when the blood K level is maintained over comparable periods at the levels (30–40 mg./100 ml.) found in adrenal insufficiency. In adrenal insufficiency the ability to dispose of injected K salts is diminished so that doses not significantly raising the blood K of normal animals are followed by a considerable, prolonged and often fatal rise.

SUMMARY.

1. A method for the determination of K in 0·2 ml. of blood has been described, and certain errors present in the ordinarily used methods pointed out.

2. The normal values found (19 cats) were: plasma 19·3, serum 20·2 and whole blood 21·4 mg. K/100 ml.

3. The results of analyses performed on 18 bilaterally adrenalectomized cats, at frequent intervals following the operation, are given; the values for blood K

vary within wide limits (11–46 mg./100 ml.) for different animals at different times after the operation. The most consistent effect is that of a rapid rise to a maximum value on the third or fourth day.

4. A method for determining K in muscle is described: an average of 0·415 % wet weight, or 1·900 % dry weight has been found for skeletal muscle of animals in the terminal stage of adrenal insufficiency, and of 275 mg./100 g. and 1·277/100 g. for myocardium. The values for wet weight are lower, and for dry weight higher, than for normal cats.

5. The significance of these findings is discussed, and it is suggested that the syndrome of cortico-adrenal insufficiency is intimately associated with a disturbance in K metabolism.

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CXCIII. THE COMPOSITION OF FORAGE CROPS.

I. RYE GRASS. (WESTERN WOLTHS.)

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MANY papers dealing with the composition and digestibility of forage crops are to be found in the literature, but the analyses are usually restricted to those few groups included in the conventional methods of agricultural analysis. These rarely extend beyond the determination of ash, or mineral constituents, crude protein, crude fibre and ether-soluble material. The sum of these analyses is far below 100 % in the case of grasses, and the difference from 100, often 40–60 %, is expressed vaguely as "N-free extractives" or "soluble carbohydrates". In the belief that more information should be obtained about the carbohydrate constituents of forage crops in general, and this ill-defined fraction in particular, detailed analyses have been undertaken using methods which have been developed in the past few years. Not only is it desirable to obtain further knowledge as to the final composition of agricultural materials used as feeding stuffs, but also to be able to follow the changes which take place during development, maturation, drying and storage. This paper records a preliminary study of the development of rye grass and its conversion into hay.

Sampling.

The samples were taken from Pastures Field, Rothamsted, in April, May and June 1935, the seed having been sown under oats the previous season. The selected area, 21 yd. sq., was divided into small plots each 1½ yd. sq. Sampling was effected by cutting with shears an area of 1 sq. yd. within the small plot, this being conveniently determined by the use of a wooden frame of that size. The rejection of margins permitted passage between the areas to be sampled and obviated the necessity of avoiding plots adjacent to one previously sampled on account of possible edge effects. At each time of sampling eight plots, selected at random in the usual way, were cut. The grass from these plots was bulked, again at random, into two samples (A and B), each containing the yield from four plots. In this way an estimate of error due to variation in composition is possible and, since all determinations were subsequently made in duplicate on these A and B samples, an estimate of analytical error has also been obtained. The number of plots cut at each time of sampling is adequate since the purpose of these investigations is a study of composition and not of yield or rate of growth. Many more plots would have to be sampled to obtain reliable figures for the latter.

Treatment of samples.

The grass when cut was bulked into A and B samples and removed to the laboratory. By hand-picking, all weeds and stubble were separated, and the sample placed in an oven at 100° for a short period to inactivate enzymes. Drying was then effected in a drying room at a moderate temperature. After chaffing the samples were ground in a high speed mill to pass 60-mesh.

Other information.

The weather conditions of early summer of 1935 were exceptional, the rainfall in the latter part of May and in June being much above average and the sunshine deficient. Between 26 April, when the first samples were taken, and 21 June, when the last were cut, the rainfall totalled 4·9 in. The sunshine in June, up to the 21st, was 92·8 hours, the average for the period being 143·4 hours. As a result the grass when mature was greener and more stemmy than is usually the case.

The whole area was mown on 22 June and the grass left for hay in the usual way, being turned once. A severe thunderstorm (0·28 in.) soaked it when partly dry. When dry, a large random sample was taken (28 June) so that information might be obtained as to the changes normally occurring in the process of hay making.

Analytical methods.

The following analyses were carried out on the air-dried material:

1. *Ash*: in an electric muffle furnace.
2. *Crude protein*: N by Kjeldahl × 6·25.
3. *Cold water-soluble fraction*: 24 hours at room temperature, nitrogen being determined in the residue.
4. *Crude fibre*: official method.
5. *Total pectin* (expressed as calcium pectate yield).
6. *Cellulose*: Norman & Jenkins [1933].
7. *Total furfuraldehyde yield*: by distillation with 12 % HCl and precipitation as the phloroglucide.
8. *Furfuraldehyde* from cellulose.
9. *Lignin* after hydrolysis: Norman & Jenkins [1934, 1], the material being pretreated with alcohol-benzene.
10. *Ether-soluble fraction*: by extraction in a Soxhlet apparatus.

RESULTS.

Some information about the rate of growth of the grass may be obtained from the dry weights of the samples (Table I), each representing the yield from 4 sq. yd.

Table I. *Yield of rye grass from 4 plots each of 1 sq. yd.*

Sample			
1	April 26	A	97·6
		B	102·8
2	May 10	A	201·2
		B	206·4
3	May 24	A	323·5
		B	290·9
4	June 7	A	494·4
		B	381·8
5	June 21	A	786·5
		B	605·3

Non-structural constituents.

Figures relating to the non-structural constituents are given in Table II and call for no special comment except in the case of the water-soluble fraction, which was very high in the younger samples. More than half of the material of the first three samples was extracted by cold water and of this the protein accounted for only a small part. Hot water was no more effective and indeed removed rather less protein, no doubt owing to coagulation. The nature of the

Table II. *Some non-structural constituents of rye grass.*

All analyses expressed as % on oven-dried samples.

Each figure is the mean of 4 analyses—duplicate determinations on 2 independent composite samples.

Sample period	Ash	Crude protein	Cold water-soluble	Protein in cold water extract	Hot water-soluble	Protein in hot water extract	Ether extract
1	9·52	10·11	53·92	3·17	52·43	3·01	3·46
2	9·11	8·56	52·28	2·90	53·02	2·39	3·42
3	7·84	7·27	54·16	2·23	55·46	2·29	6·34
4	7·66	7·15	46·21	2·45	47·76	2·64	4·12
5	7·50	4·72	37·21	0·89	38·27	0·89	4·84
Hay	7·58	5·46	32·07	2·65	31·29	2·12	2·62
*S.E. ± (sampling error)	0·30	0·09	0·55	0·12	0·63	0·22	0·15
S.E. ± (analytical error)	0·19	0·09	0·36	0·10	0·30	0·08	0·44
Analytical error % ±	2·3	1·2	0·8	4·2	0·6	3·8	10·6

* S.E. = Standard error. Sampling error includes analytical error.

Since each figure in the table is the mean of 4 analyses, the sampling and analytical errors per sample are obtained by doubling the errors given.

non-nitrogenous water-soluble fraction will be dealt with later. The increase in crude protein content on drying for hay was of course due to carbohydrate losses, and the apparent increase in crude protein passing into solution in cold and hot water in the same sample to some protein degradation resulting in the production of soluble products. This will be discussed later.

Structural constituents.

Analyses of some structural constituents are given in Table III. With increasing age the cellulose content rose rapidly, particularly in the samples 4 and 5. For comparison, crude fibre determinations were made by the official method. The deficiencies of the crude fibre figure have been dealt with in detail

Table III. *Some structural constituents of rye grass.*

All analyses expressed as % on oven-dried samples.

Each figure (except N in lignin) is the mean of 4 analyses—duplicate determinations on 2 independent composite samples.

Sample period	Cellulose	Furfuraldehyde from cellulose	Xylan in cellulose	Total furfuraldehyde yield	Furfuraldehyde from polyuronides	Lignin	N in lignin	Crude fibre	Calcium pectate
1	20·89	2·63	4·11	7·29	4·66	4·63	0·29	18·76	0·91
2	20·45	2·75	4·30	7·73	4·98	4·37	0·28	18·17	0·41
3	23·15	3·99	6·19	7·77	3·78	5·08	0·27	17·08	0·52
4	29·69	5·44	8·45	9·85	4·41	6·30	0·27	21·54	0·60
5	36·30	7·09	11·02	11·31	4·22	8·48	0·28	26·34	0·42
Hay	40·85	7·61	11·80	13·15	5·54	9·32	0·28	31·60	0·19
S.E. ± (sampling error)	0·31	0·14	0·21	0·16	0·24	0·17	*	0·42	0·05
S.E. ± (analytical error)	0·21	0·09	0·14	0·22	*	0·07	*	0·33	0·04
Analytical error % ±	0·76	1·92	1·79	2·4	5·3	0·90	*	1·54	7·85

* Not determined.

elsewhere [Norman, 1935, 1] but it is sufficiently apparent from the rye grass samples that the cellulosic structure of the plant is underestimated in an irregular manner in the fibre determination. Whereas in sample 1 the crude fibre figure accounts for about 90% of the cellulose, in samples 4 and 5 only 72% is estimated. A contributory factor in this is the increase of xylan in the cellulose with increasing age. This change is more obvious if the xylan be expressed as a percentage of the cellulose containing it (Table IV).

Table IV. *Xylan content of rye grass at different ages. Expressed as % of the cellulose.*

Sample	Xylan
1	19·7
2	21·0
3	26·7
4	28·5
5	30·4

Similar, but not entirely regular, increases in the content of xylan associated with the cellulose have been found in the barley plant by Norman [1933] and by Phillips & Goss [1935]. It is not easy to see why there should be this change in the nature of the cellulosic framework of the plant with increasing age. The glucose and xylose units forming the cellulose and xylan are presumably laid down together by the same mechanism, the balance altering in favour of the pentose as the plant becomes more mature. The pentose metabolism of plants is little understood as yet and provides a number of such problems. It has previously been pointed out that a calculation of total pentosans from the furfuraldehyde yield of the whole material is misleading because of the changing proportion of xylan in association in the cellulose [Norman, 1933] and this fact is again evident from the rye grass results. The total furfuraldehyde yield increased with age, but whilst in the youngest material 26% of the total was derived from the xylan in the cellulose, the amount from this source increased to 62% in the mature material. The nature of these changes may be seen from Fig. 1. Since the direct determination of hemicelluloses is unsatisfactory, the furfuraldehyde not from cellulose is taken as a measure of this group of encrusting polyuronides. No correction has been made for the furfuraldehyde derived from pectin, since the pectin content was quite low throughout. The proportion of encrusting polyuronides apparently remained much the same over the whole period of growth. Information as to the actual amount is not given by these analyses. However, the furfuraldehyde yields of hemicellulose preparations that have been obtained indicated that the factor for conversion of furfuraldehyde from this source to polyuronide is a little over 2.

Since the ripening of grass is always said to be accompanied by lignification, and since the extent of lignification considerably affects digestibility, the lignin contents of these samples are of especial interest. The determination of lignin has been a matter of much research lately, and whilst some of the disturbing factors have been recognized, it cannot be said that a method of general applicability has been evolved. In the presence of protein, errors are introduced by the linkage of protein fission products with the lignin [Norman & Jenkins, 1934, 2]. Some workers have corrected for this disturbance by determining the N present in the lignin product, calculating this as protein and subtracting. Such a correction is not justified, and until such time as a method which shall be free from protein interference is devised, it is preferable simply to record the N found in the lignin. The pretreatment advocated by Norman & Jenkins [1934, 1] for reducing the errors due to the presence of pentose groups does at the same time considerably reduce the protein disturbance. The lignin figures given in Table III were obtained on materials which had been given this pretreatment following extraction with alcohol-benzene, as usually advocated for woods. The apparent lignin residue contained over 6 % N on an ash-free basis in the case of the youngest sample. It was curious however that the actual amounts of N retained by the lignin were almost identical in each case although the actual yield of apparent lignin increased progressively with age, the final sample containing nearly double that of the youngest. The error due to protein may therefore be taken to be more or less constant in this series and the increase in lignin content to be from about 3 % in the youngest sample to about 7 % in the most mature. The importance of the alcohol-benzene extraction for the removal of fatty and waxy compounds in the case of such green materials was made evident when lignin determinations were carried out on the ether-extracted residues, an acid pretreatment being given in both cases. For comparison, the determination was also made without a solvent extraction (Table V).

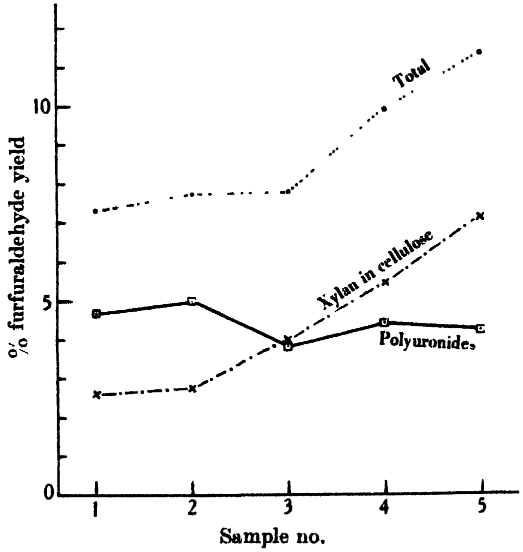

Fig. 1. Distribution of furfuraldehyde yield from rye grass samples.

Table V. *Effect of solvent extraction on lignin determinations.*

All results expressed on 100 g. oven-dry material.

Rye grass sample	Alcohol-benzene extraction: Apparent lignin	Alcohol-benzene extraction: N in lignin	Ether extraction: Apparent lignin	Ether extraction: N in lignin	No solvent extraction: Apparent lignin	No solvent extraction: N in lignin
1	4·63	0·29	*	*	6·58	0·25
2	4·37	0·28	5·27	0·33	6·37	0·21
3	5·08	0·26	5·99	0·31	5·72	0·21
4	6·30	0·27	6·79	0·32	7·14	0·20
5	8·48	0·28	9·21	0·28	9·73	0·19
Hay	9·31	0·28	11·15	0·32	10·64	0·20

* Not determined.

It is evident that a further examination of the effect of solvent extraction on the determination of lignin in green plant materials is called for. Although treatment with alcohol-benzene resulted in the lowest yield of lignin residue, the nitrogen in the lignin was actually increased by extraction with either ether or alcohol-benzene. The differences between ether-extracted and alcohol-benzene-extracted samples are greater than could be accounted for by the higher N content of the lignin from the former.

Water-soluble constituents.

The astonishingly high content of material soluble in cold water in these samples led to an investigation of the nature of the substances removed. The major constituent of the extract appears to be a fructose anhydride or laevan. The presence of such fructose polysaccharides in members of the Gramineae has been reported at various times, and recently Challinor *et al.* [1934] have studied the laevan from rough-stalked meadow grass. The yield of pure laevan obtained by them was less than 1 % of the dry weight of the grass taken. Buston [1934], in the course of an investigation on the polyuronides of grasses, determined the amount of reducing sugar in the aqueous extract of young grass samples before and after hydrolysis with 1 % H_2SO_4 for 40 min. The increases on hydrolysis, by no means all necessarily due to fructose, suggest that the fructosan content of the samples investigated by him was low, except perhaps in Crested dog's tail (*Cynosurus cristatus*). Preparations of fructosan have also been obtained from rye [Kizel & Kretovitsch, 1934] and from barley [Archbold & Barter, 1935].

The accurate determinations of the fructosan in the rye grass samples presented difficulties which have not been entirely overcome. It was first determined that an aqueous extract could be hydrolysed readily by low concentrations of acid and that 0·5 % oxalic acid gave no increase in fructose after heating for 30–45 min. Even at such a dilution, hydrolysis was extremely rapid in the first 15 min. Determinations were carried out first on cold aqueous extracts of the samples, the extraction being made for 24 hours. This was found to give an incomplete extraction of fructosan and accordingly a second treatment for the same time was given. After clearing with a small amount of basic lead acetate and de-leading with sodium phosphate, the total reducing value was determined by the Shaffer-Somogyi [1933] micro-method, and the aldoses by hypoiodite oxidation for 2 hours in a refrigerator. Similar determinations were made after hydrolysing the extract by boiling with 0·5 % oxalic acid in a water-bath for 1 hour, conditions which were found to result in the complete hydrolysis of the fructosan. The results are given in Table VI, the figures obtained by the copper method being calculated as fructose and those by the iodine method as glucose.

Table VI. *Carbohydrate in aqueous extracts of rye grass samples.*

Results calculated on the basis of 100 g. oven-dry grass.

	First extraction				Second extraction			
Sample	Total sugar before hydrol.	Total sugar after hydrol.	Apparent glucose before hydrol.	Apparent glucose after hydrol.	Total sugar before hydrol.	Total sugar after hydrol.	Apparent glucose before hydrol.	Apparent glucose after hydrol.
1	3·10	27·47	9·66	9·96	0·06	0·98	0·56	0·70
2	7·00	30·88	8·12	9·18	0·11	0·77	0·07	Trace
3	6·37	35·15	9·20	10·49	0·05	1·16	0·50	0·56
4	4·28	26·66	7·97	8·50	0·11	1·06	0·47	0·42
5	6·54	21·77	7·02	6·69	0·12	1·09	0·47	0·47
Hay	5·60	15·68	7·83	7·83	0·07	0·34	0·50	0·25

Two important facts are at once apparent. First, the increase in reducing sugar produced by hydrolysis is due almost solely to the liberation of fructose, since the apparent glucose figures show little difference after hydrolysis. Second, the extract must contain oxidizable material unaffected by the copper reagent, but easily oxidized by hypoiodite, because the total reducing value by the copper reagent is far lower than the apparent glucose content by hypoiodite oxidation. This is, of course, a common observation with plant extracts and makes it impossible to get true figures for fructose and fructosan contents by this method. Hot water extraction for 1 hour gave figures for total reducing sugar very similar to the sum of the cold extracts with the difference that the amounts of apparent glucose after hydrolysis determined by hypoiodite oxidation were a little higher. Direct hydrolysis of the fructosan in the grass was effected by heating for 30 min. with 5 % oxalic acid in a water-bath. As far as could be judged, the results obtained indicate that hot aqueous extraction or repeated cold aqueous extraction does result in complete removal of the fructosan. To avoid difficulty due to the presence of aldose sugars and non-sugar reducing substances, determinations were carried out by the copper reagent on extracts which were first oxidized by hypoiodite, the excess iodine being removed with sulphite. The procedure adopted was similar to that recently described by Van der Plank [1936]. The grass samples, finely powdered, were extracted for 30 min. in a boiling water-bath, and a portion of the extract, after clarification, hydrolysed by boiling with 2 % oxalic acid for 30 min.

5 ml. portions of the unhydrolysed and hydrolysed extracts were treated with 2 ml. *N*/10 iodine (containing 34 g. KI per litre) and 1 ml. *N*/4 NaOH and kept in a refrigerator just above freezing point for 2 hours. After acidification with 1 ml. *N*/3 H_2SO_4 the excess iodine was removed by titrating with dilute sulphite. The reducing value of this solution was then determined in the usual way with the Shaffer-Somogyi reagent. As a result of the pretreatment with hypoiodite, considerable quantities of iodide are present which appreciably affect the apparent reducing value of fructose. The values for the fructose content must therefore be taken from a curve specially determined for the quantity of iodide that is present. Unless this be done the error introduced is over 10 %. The results obtained are given in Table VII. The fructose or fructosan present passed through a sharp peak and then rapidly declined as maturity approached. A further considerable fall took place in the process of hay making. The intervals at which the samples were taken were not sufficiently short to provide a full picture of the changes in the fructosan content. It is however quite evident that this polysaccharide must be the chief carbohydrate reserve of rye grass, and, because of the high availability of fructose, must be also of great nutritional importance. The method of drying adopted was such that some slight hydrolysis of fructosan to fructose might take place. A more detailed investigation of the

Table VII. *Fructosan in rye grass samples.*

Results calculated on the basis of 100 g. oven-dry grass.

Sample	Before hydrolysis as fructose	After hydrolysis as fructose	Fructosan as fructose
1	1·06	28·21	27·15
2	3·00	26·62	23·62
3	3·10	37·53	34·43
4	2·69	25·33	22·64
5	3·73	18·87	15·14
Hay	5·23	14·19	8·96

distribution of this polysaccharide is in progress. Young wheat plants have also been found to contain fructosan, the amount decreasing rapidly as the ears form (unpublished).

Hydrolysis of rye grass fructosan.

A crude preparation of fructosan was obtained from an aqueous extract of young dried rye grass (collected 17 May). Proteins were removed with basic lead acetate and lead with phosphate. After concentration to a small volume *in vacuo*, inorganic salts were precipitated by the addition of alcohol to give a concentration of 47 %. After further concentration, the solution was poured into a large excess of absolute alcohol giving a creamy white precipitate, which was immediately filtered off. Purification of the precipitate was attempted by several means, including the baryta fractionation described by Archbold & Barter [1935], but no method was very successful. The crude precipitate had a specific rotation $[\alpha]_D -40^\circ$ approximately and very slight reducing properties. After hydrolysis for 15 min. with 0·5 % oxalic acid the sugar produced amounted to 87 % on an ash-free basis, 84·4 % being fructose. This polysaccharide was very susceptible to hydrolysis and yields of sugar of a similar magnitude were obtained on heating for 30 min. with 0·05 % oxalic acid; using 0·025 % oxalic acid the sugar produced did not exceed 58 %. Slight hydrolysis was effected on prolonged boiling with water, 19·0 % sugar being obtained after 1 hour and 20·8 % after 3½ hours.

The changes occurring during hay making.

No direct determinations of the total losses occurring during hay making were made, as the whole area was cut. Some indication of the magnitude of these losses may, however, be obtained by comparing in the mature sample and the hay the percentage contents of constituents such as cellulose and lignin which would be unlikely to be reduced by the treatment. Such a comparison suggests that the losses were in the neighbourhood of 10 %. Of this more than half was fructosan, presumably as a result of respiration, or enzymic conversion of the polysaccharide into fructose which would be liable to be washed out by rain. As evidence of the breakdown it is significant that the hay contained the highest percentage of free fructose. Some losses of ash and ether-soluble material also occurred. There does not appear to have been any loss in the nitrogenous constituents as judged by the total N figures, but extensive changes must have taken place because the cold water-soluble N increased from approximately 20 to 50 % of the total. The xylan in the cellulose was slightly reduced, with a concurrent increase in furfuraldehyde from the polyuronides. The pectin content, though low in the mature grass, fell sharply.

Discussion.

The system of analysis described in this paper though not complete accounts far more satisfactorily for the various constituents of grass than conventional methods. It is imperfect in that a reliable method for the determination of the polyuronide hemicelluloses has not been found. Buston [1934] attempted this determination by direct preparation and found the apparent hemicellulose content of various grasses by this method to be 16–20 %. His procedure is, however, very laborious and is open to certain criticisms [Norman, 1935, 2], the chief perhaps being that any alkaline extraction also removes cellulosan from the cellulose, with the result that the product does not only consist of encrusting polyuronides. An attempt to avoid this difficulty will be described elsewhere.

In the case of sample 5, only 43·4 % of the grass is accounted for by the conventional methods, leaving 56·6 % to be described as soluble "carbohydrates". Excluding any estimate of the hemicelluloses, the analyses given in this paper on the same sample total to over 80 %. The presence of a considerable amount of soluble carbohydrates in the form of fructosan is now established and the role of this in nutrition will have to be examined. Presumably it is readily utilized by the animal, though the action of digestive enzymes on this polysaccharide has not been studied. Archbold & Barter [1935] found that their fructosan preparations were but slowly attacked by invertase. The position of fructosan in the metabolism of the plant can as yet be only a matter of speculation. In the young plant fructosan is produced more rapidly than cellulose, but later cellulose production predominates. This is shown in Table VIII in which the chief constituents in each sample are expressed approximately as percentages of that present in the final sample, the increases in yield being taken from the means of the figures in Table I.

Table VIII. *Amounts of various constituents as percentages of that present in final sample* (*5*).

Sample	Dry matter	Fructosan	Cellulose	Lignin	Crude protein
1	14·4	22	8	8	31
2	29·4	41	17	15	54
3	44·1	87	28	26	69
4	63·0	84	52	48	96

Some of the advantages that have been ascribed to the higher protein content and greater digestibility of the cellulose of young grass may in fact be due to its much higher content of fructosan, if other species of grass are at all similar to rye grass.

SUMMARY.

1. Samples of rye grass cut at fortnightly intervals have been analysed, particular attention being given to the structural constituents.

2. The contents of cellulose and lignin increased rapidly as maturity approached. The percentage of xylan in the cellulose also increased with age. The polyuronide hemicelluloses, as judged by furfuraldehyde yield did not exhibit any regular increase and were lower in the mature grass than in the young grass.

3. A water-soluble fructosan, or laevan, was found in considerable amounts in the younger samples, reaching at one stage over 37 % expressed as fructose. The percentage content of this polysaccharide fell rapidly on maturity.

4. Losses in hay making were of the order of 10 %, mostly accounted for by loss of fructosan.

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CXCIV. EFFECTS OF COLCHICINE AND RELATED SUBSTANCES ON CELL DIVISION.

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The effects of the alkaloid colchicine as a mitotic poison were first described by Lits [1934] and Dustin [1934], and the nature of its action has been further studied recently by Ludford [1936]. Amoroso [1935] has reported experiments on the inhibition of tumour growth in mice and a dog by injection of colchicine, although uniform results have not been obtained by subsequent workers. In common with a number of apparently unrelated substances, notably the cacodylates, it has been shown (Lits, Dustin, Ludford) to arrest cell division during metaphase and to maintain cells thus affected in this phase of mitosis for several hours following administration; this occurs both in tissues *in vivo* and in explanted tissue. It is, of course, most readily seen in tissues in which the mitosis rate is normally high, notably in the glands of the intestine, and it occurs as well in malignant tumours and in explants from them. It has been possible to show [Brues, 1936] in the case of the regenerating rat liver (in which the normal rate of cell division is known with reasonable accuracy) that under suitable conditions the number of mitoses seen in arrested metaphase after administration of colchicine over a given length of time is equal to the number of mitoses which would normally have occurred and gone on to completion during that time. The present study has been made in an attempt to determine the effects on mitosis of various compounds derived from colchicine, to assess the importance of the various molecular groupings of colchicine and to determine whether similar mitotic effects may be shown with colchicine derivatives which are devoid of the high toxicity of the parent alkaloid.

It has been essential to select as a test-object a tissue with a fairly high normal rate of mitosis in which the abnormal arrested metaphase can be unequivocally distinguished from the normal metaphase. Experimental tumours fulfil the first of these criteria, but owing to the frequency of abnormal mitotic figures it is often impossible to say whether a given mitosis shows the toxic effects of a mitotic poison or not. Moreover, we have observed in tumours that many cells in apparently normal later stages of division are seen after effective doses of the drug have been given and, at least in the case of certain transplanted sarcomata (probably owing to inadequate blood supply), the effect may be confined to the borders of the tumour and in some sections missed altogether.

The most satisfactory tissue for our purpose has been liver in the process of restoration following subtotal hepatectomy. In the case of the rat, the average mitosis rate during the period of rapid regeneration is nearly as great as that in most experimental tumours; and histological study is facilitated by the large size of the hepatic cells and by the highly characteristic distribution of the chromosomes under the influence of a mitotic poison. In the normal hepatic cell mitosis the chromosomes form a very compact group shortly after the disappearance of the nuclear membrane during prophase and remain so throughout the division: except in prophase it is impossible to distinguish and count the individual chromosomes (Pl. III, fig. 1). On the other hand, after parenteral administration of a suitable dose of colchicine, it can be seen that the chromosomes scatter widely throughout the cell shortly after disappearance of the nuclear membrane, as if they repelled one another, and it is often possible to count them (Pl. III, figs. 2 and 3). In addition, the cell in this arrested stage has the rounded border characteristic of a cell in mitosis and in most cases the cytoplasm stains much more lightly with eosin. Since we have never seen (except under the influence of a very small dosage) these abnormal figures in the same section with normal metaphases and later stages of mitosis, it is easily possible to distinguish the abnormal picture from the normal one. An appropriate dose of sodium cacodylate gives essentially the same picture in this organ.

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Technique.

Using the customary aseptic precautions partial hepatectomies have been performed on a series of rats weighing 100–200 g. by a method described previously [Higgins & Anderson, 1931]. According to this method, the main and lateral lobes are removed, leaving approximately 30 % of the liver intact. This remnant has been shown [Brues *et al.* 1936] to begin increasing in size at once, whilst mitoses begin to appear in large numbers after 24 hours. We have therefore administered the substances to be tested 24–30 hours post-operatively, when mitosis is usually at its height. The animals were then killed for microscopic examination of tissues after a further interval of 6–18 hours. The drugs were given subcutaneously. Intraperitoneal administration seemed unwise since often a little ascites is seen at the time of autopsy. Microscopic examination was made on tissue fixed in 10 % formalin, sectioned in paraffin and stained with iron haematoxylin and eosin, or for purposes of rapid results on tissue smears stained with Leishman's stain, differentiated rapidly in 95 % alcohol and mounted in balsam.

Upon administration of colchicine in aqueous solution subcutaneously, the greatest numbers of arrested mitoses were seen with a dosage of 0·1–0·2 mg. per 100 g. body weight. With doses of 0·02–0·05 mg. partial effects were seen in which the numbers of arrested mitoses were smaller, whilst many figures were seen in which some of the chromosomes had scattered, while the rest remained in a group. When large doses (0·5–10 mg.) were given, fewer figures were seen than with the smaller optimum amounts. In the case of these large doses, which usually are lethal if the experiment is prolonged to 24 hours, it was found that the number of abnormal figures seen in animals receiving the injections less than 24 hours after operation was much smaller (0–0·2 %) than in animals injected more than 24 hours after operation (1–3 %). Since these percentages are quite similar to the percentages of mitoses seen in the normally regenerating liver before and after the 24-hour interval, it seems likely that large doses of colchicine prevent cells from entering mitosis, so that the abnormal figures seen represent the cells which were already in mitosis at the time of administration. This may also be the case in a certain few rats receiving doses of 0·1 and 0·2 mg. in which only a few abnormal mitoses could be found. In no case receiving an effective dose (0·1 mg. or more) of colchicine more than 24 hours after operation did we fail to find some abnormal figures.

Since the percentage of abnormal mitoses seen is therefore not wholly dependent upon dosage, we have for purposes of assay tried to determine a

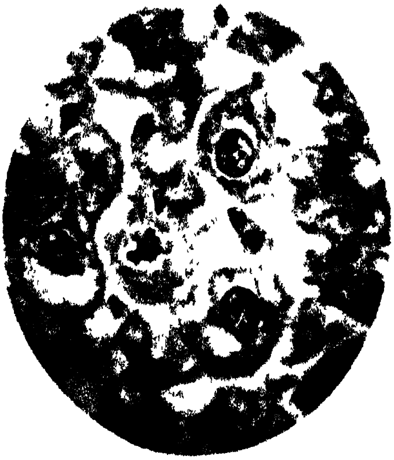


Fig. 1. Normal metaphase (indicated by arrow) in regenerating liver 30 hours after operation. ×600.

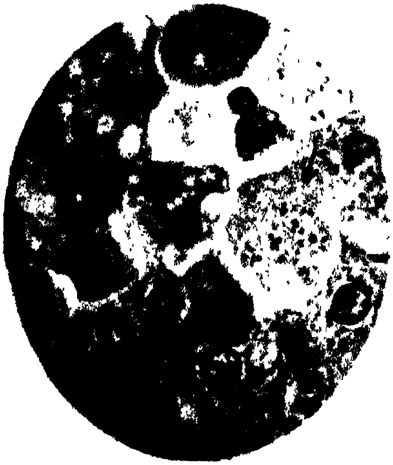


Fig. 2. Abnormal mitoses in regenerating liver 2 days after operation and 8½ hours after colchicine treatment (0·1 mg. subcutaneously). One abnormal figure is indicated by an arrow. ×600.

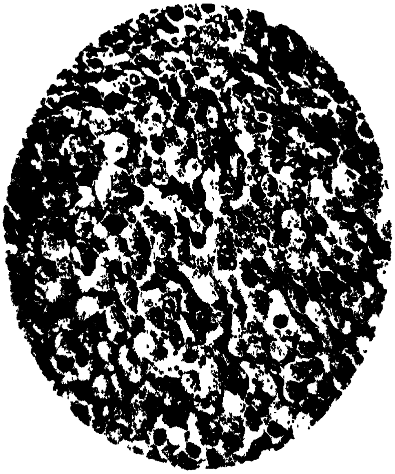
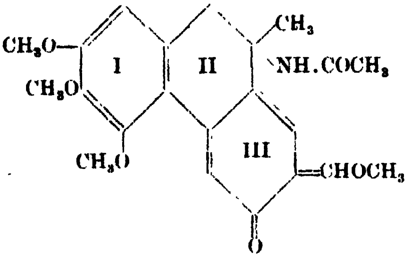


Fig. 3. Low-power view of the liver shown in Fig. 2 showing large numbers of arrested mitoses. ×95.

"minimum dosage" which gives partial abnormalities of mitosis, above which the typical picture of wholly scattered chromosomes is seen and below which mitoses appear normal. All dosages are expressed in mg. of substance per 100 g. body weight.

Results.

Colchicine has as its nucleus a partially hydrogenated phenanthrene ring, and Windaus [1924] has proposed the following structure for it, the only features which he regards as uncertain being the positions of the two substituents in ring III.



The colchicine employed in these experiments was obtained from the Hoffman La Roche Laboratories. Since this substance contains about 25% chloroform of crystallization, a few rats were injected with amorphous colchicine, which contains no chloroform, with entirely similar results as far as the cytological picture was concerned.

Colchicine was dissolved in 0·9% saline solution before administration. When large quantities were used, it was first dissolved in a minimum amount of alcohol, in order to facilitate solution in water. In order to control the use of alcohol in the administration of this and other substances, comparable amounts of alcohol alone have been injected into animals during hepatic regenerations, without any resulting abnormalities of mitosis being detectable.

A small series of rats were given subcutaneous injections of colchicine suspended in sesame oil, in the hope of retarding the absorption. If there is any real difference in the action of the oil solution, it appears to increase the mitotically effective dose and to decrease the minimum toxic dose, as appears in Table I.

Colchiceine [Zeisel, 1886], in which the $=CHOCH_3$ group of colchicine has been hydrolysed to $=CHOH$, was employed in saline solution. It is interesting that the minimum lethal dose of colchiceine (3 mg.) often kills within 2 hours of the time of administration, whilst the much more toxic substance colchicine, even in a dosage of 10 mg., does not kill before 8 hours after administration.

Colchicine salicylate, which is a simple molecular compound of colchicine with salicylic acid, and is in common medicinal use, appears similar to colchicine in its action and dosage, as might be expected.

Octahydrocolchicine [Windaus, 1924], which is obtained by catalytic hydrogenation of colchicine, is a derivative of *as*-octahydrophenanthrene in which the methoxymethylene group and the carbonyl group are both reduced. The specimen was prepared by Dr E. Boyland and used in saline solution.

Dimethyl- and trimethyl-colchicinic acids [Zeisel, 1888] are products of hydrolysis which contain a free amino-group in ring II and a free hydroxymethylene group in ring III. In trimethylcolchicinic acid the three methoxyl groups in ring I are preserved intact, but in dimethylcolchicinic acid one of these is hydrolysed to hydroxyl. These two compounds were used in saline solution, neutralized with $N/10$ NaOH. It will be seen that these derivatives alone have been ineffective in any sublethal dosage.

Table I.

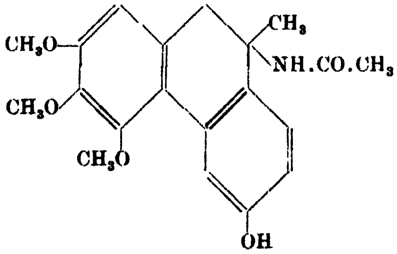
Substance	No. of animals	Minimum effective dose mg./100 g.	Optimum dose (where determined) mg./100 g.	Average lethal dose mg./100 g.
Colchicine (aqueous)	55	0·02	0·1	0·5
Colchicine in oil	5	0·1	0·2	0·2
Colchicine salicylate	5	0·05	0·1	1·0
Colchiceine	6	0·8	3·0	3·0
Octahydrocolchicine	3	3·0	10·0	10·0
N-Acetylcolchinol	6	0·9	3·0	20·0
N-Acetylcolchinol methyl ether	5	8·0	—	12·0
Colchinol methyl ether hydrochloride	4	6·0	—	20·0
Corresponding carbinol*	6	7·5	10·0	†
N-Acetyliodocolchinol	4	10·0	—	20·0
Dimethylcolchicinic acid	5	—	—	10·0
Trimethylcolchicinic acid	5	—	—	20·0

* This carbinol, which will be described elsewhere (Cohen & Cook), was obtained by the action of nitrous acid on colchinol methyl ether.

The "minimum effective dose" is the smallest dose which gives obvious abnormalities of mitosis. The "optimum dose" is the average amount which gives a maximum number of abnormal mitoses, i.e. which is effective in stopping mitosis in metaphase but which does not prevent cells from entering mitosis.

† The toxic dose of this substance is probably higher than 20 mg., but there was not sufficient material to determine this dosage.

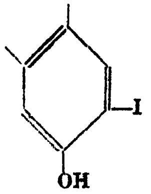
N-*Acetylcolchinol* [Windaus, 1919]. This is a more profoundly modified colchicine derivative, and on the basis of Windaus's structure for colchicine would be represented by the formula:



This was dissolved in a small amount of alcohol and made up by the addition of saline solution. The *methyl ether* [Windaus, 1919] of this compound, which was less soluble in both alcohol and water than the free phenol, was utilized in the same way. *Colchinol methyl ether hydrochloride* [Windaus, 1919] is the hydrochloride of the free base arising from hydrolytic removal of the acetyl group, and was dissolved in saline solution by neutralization with $N/10$ NaOH.

The *carbinol* obtained by the action of nitrous acid on this base was dissolved first in alcohol; with the addition of saline solution a fine colloidal suspension of the substance was obtained for injection.

N-*Acetyliodocolchinol*, an intermediate in the preparation of *N*-acetylcolchinol, in which ring III is as follows:



was injected in fine suspension.

Discussion.

Although the colchicine molecule contains a variety of molecular groupings (e.g. methoxyl, methoxymethyleneketone, acetylamino) it cannot be said from the results of this investigation that any single group is essential for the mitotic inhibiting action of colchicine. The compounds examined include colchicine derivatives in which the methoxymethylene group has been first hydrolysed to hydroxymethylene, then replaced by iodine, and finally completely eliminated, the last two stages being accompanied also by modification of the ketonic group (conversion into a phenolic hydroxyl group). Also, some of the compounds examined have had the acetylamino-group intact, others have had this group hydrolysed to the free amino-group, while in yet another case the amino-group is replaced by hydroxyl. All of these derivatives have shown activity. The inactivity of dimethyl- and trimethyl-colchinic acids is of interest. In the former case the suppression of activity might perhaps be attributed to demethylation of one of the three methoxyl groups in ring I, but trimethylcolchicinic acid appears to be anomalous, for the only modifications in the colchicine molecule are (*a*) hydrolysis of the methoxymethylene group to hydroxymethylene and (*b*) hydrolysis of the acetylamino-group to the free amino-group, whereas it has been shown that neither of these changes is necessarily accompanied by loss of activity. Possibly the inactivation of trimethylcolchicinic acid is associated with the presence of the basic grouping and also the strongly acidic hydroxymethylene group in the same molecule. The lack of specificity suggested by these results appears to warrant the examination of synthetic compounds of analogous structure, and experiments on these lines are in progress.

It will be seen that no substances are effective on mitosis in the small doses required in the case of colchicine itself, and that the toxicity roughly follows the same relative dosage with different compounds, except in the case of the colchicinic acids (which are ineffective on mitosis), and possibly in the case of the nitrogen-free carbinol, in which the lethal dose is as yet undetermined. However, the fact that the lethal effect of colchicine appears only after several hours, when the mitotic effect is beginning to wear off, suggests that the two effects may be dissociated, and this question requires further investigation.

There is obviously a wide gap between the effective and lethal doses of colchicine (and its salicylate) and those of the other substances. Some of the other differences of dosage may, however, depend upon variations in solubility and absorbability.

Summary.

A number of derivatives of colchicine have been investigated with regard to their effects as mitotic poisons. The test object was the regenerating rat liver, in which the arrested mitosis is conspicuous and is easily distinguished from the normal mitosis.

Colchicine, injected subcutaneously in aqueous or oily solutions, produces arrest in metaphase of a maximum number of mitoses only within certain limits of dosage. Larger doses appear to prevent cells from entering mitosis, whilst in smaller dosage partial effects are seen.

Colchicine, octahydrocolchicine, *N*-acetylcolchinol and four derivatives of the latter all produce similar effects to colchicine, but in considerably higher dosage. Dimethyl- and trimethyl-colchicinic acids have been ineffective in any sublethal or lethal dosage.

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CXCV. PHOSPHORYLATION IN LIVING YEAST.

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EULER & ADLER [1935] recently described the preparation from yeast of a cell-free enzyme, "heterophosphatese" which catalyses the transference of phosphate from (muscle) adenosinetriphosphate to glucose or fructose with the formation of a hexosephosphate. Later Meyerhof [1935] showed that preparations of hexokinase contain "heterophosphatese". The natural inference from these experiments is that adenosinetriphosphate may act as a phosphate carrier in alcoholic fermentations by living yeast, in a manner analogous to its role in muscle glycolysis [see Meyerhof & Kiessling, 1935]. This receives material support by the isolation of the triphosphate in crystalline form from the trichloroacetic acid extract of fresh yeast by Wagner-Jauregg [1936] who concluded that it was identical with that of muscle. The amount obtained (60 mg. from 100 g. yeast) contains approximately 2 % of the total phosphorus of the yeast.

It seemed possible that the amount of adenosinetriphosphate present in yeast might be increased during the fermentation of sugar by synthesis from adenylic acid, and this question was investigated. Judging by the analysis of the sparingly soluble barium salts no increase in the triphosphate content was found if the yeast were extracted with trichloroacetic acid whilst fermenting sugar rapidly, but it was noticed that such extracts contained less orthophosphate than similar extracts from resting yeast. It is very generally inferred that alcoholic fermentation by living yeast involves esterification of orthophosphoric acid with carbohydrate, similar to that observed in cell-free fermentations, though there is no direct evidence in support of this view. The decrease in orthophosphate has therefore been investigated more fully, and it has been established that at the onset of fermentation of sugar by living yeast the acid-soluble orthophosphate rapidly decreases to a fairly constant level and rises again to the original level when the sugar is consumed.

Whilst this work was in progress Lewitow [1936] showed that as a result of the fermentation of sugar both the (acid-soluble) ortho- and pyro-phosphate contents of living yeast were decreased and came to the conclusion that pyrophosphate acts as a phosphate carrier in alcoholic fermentation in the same manner as adenosinetriphosphate in the glycolysis of animal cells. His experimental findings are open to criticism on several points. The routine deproteinization with trichloroacetic acid was preceded by 5 minutes' boiling of the yeast suspension, which materially alters the phosphorus distribution (see Table IV). The value for pyrophosphate was calculated from the hydrolysis curve in N HCl at 100° according to the equation

$$\text{Pyro-P} = \text{P}_{7\,\text{min.}} - [\text{P}_{0\,\text{min.}} + \tfrac{7}{8}\,(\text{P}_{15\,\text{min.}} - \text{P}_{7\,\text{min.}})]$$

[Braunstein, 1931] which does not provide an accurate correction for the presence of other phosphoric esters. Finally the implication from the statement "Unter Zuckerzusatz, d.h. in gärender Hefe" that the yeast was still fermenting after 5 hours' incubation is correct only if sufficient sugar were present, which appears doubtful, and no evidence was offered that the changes induced by the fermentation were reversed by its cessation. His conclusion that during fermentation the orthophosphate decreases in amount has been fully confirmed in the present work, but no evidence was found to support the view that pyrophosphate acts as a phosphate carrier.

EXPERIMENTAL.

Phosphorylation in living yeast.

The yeast was washed with water, filtered and pressed. 1 g. samples of pressed yeast were weighed into fermentation flasks, water or sugar solution (total volume 12 ml.) being added as desired; the flasks were placed in a thermostat at 30° and connected to a nitrometer for measurement of the CO_2 evolved. The enzyme action was stopped by the addition of 3 ml. 25 % trichloroacetic acid. Phosphate estimations were carried out by Briggs's method on aliquot portions of the trichloroacetic acid filtrate, after appropriate treatment, to obtain values for

(*a*) total acid-soluble phosphate, after ashing with H_2SO_4 and H_2O_2;

(*b*) orthophosphate, by direct estimation;

(*c*) "labile" phosphate by calculation from the hydrolysis curve in *N* HCl at 100° using Lohmann's equation.

$$\text{“Labile” P} = \Delta_{7\,\text{min.}-0\,\text{min.}} - \Delta_{30\,\text{min.}-7\,\text{min.}},$$

which provides a more accurate correction for the presence of other phosphoric esters than that of Braunstein;

(*d*) organic phosphate, calculated as the difference between total phosphate and the sum of the inorganic and labile phosphate values.

Details of the experiments will be found in Tables I and II. It may be emphasized that no phosphate was added to the fermentation flasks. The variation in the values in duplicate experiments due to the sampling of the yeast and the accuracy of the estimations was of the order, for orthophosphate, ±0·02 and for total, labile and organic phosphate, ±0·05 mg. P per g. yeast.

The experiments recorded in Table I were carried out with brewery pressed yeast (mild ale) or with yeast grown four days in yeast extract in the laboratory from an inoculum of brewery yeast. These batches were nominally living yeast, but it was found that rather a large proportion (6–20 %) of the cells were stained after standard treatment with methylene blue (1 drop of 0·1 % methylene blue to 17 drops of yeast suspension containing 40,000 cells per $mm.^3$). Whether or not such cells are to be considered dead in the sense that they will no longer reproduce, their capacity to be stained must reflect a significant difference in condition, whatever the immediate cause. The possibility and even probability thus arises that the fermentations induced by such cells may be as qualitatively different from that of unstainable cells as is the fermentation of yeast juice. So that for example an accumulation of hexosephosphate during CO_2 production by a small proportion of the cells might be interpreted as a small but definite change in the phosphorus distribution in all the cells. Moreover, it was realized that a temperature above 25° is generally considered pathological for yeast, so that incubation at 30° might produce fallacious results even though the initial percentage of cells stainable with methylene blue was low.

Through the courtesy of Mr Julian Baker, yeast was obtained from the brewery vats two days after pitching, at a stage of maximum growth and with a very low count (0–5 %) of stainable cells. In order to eliminate from consideration the small changes in phosphorus distribution which take place when yeast

Table I. *Phosphorylation in living yeast at 30°.*

Treatment of yeast	Total incubation time min.	% cells stained	Type of fermentation	Acid-soluble phosphorus. mg. per g. yeast Total P	Ortho-P	Labile P	Organic P
Y. 9. Brewery press yeast 1 day old.							
1. +10 ml. H_2O; not incubated	0	18	—	2·48	0·71	1·18	0·59
2. +10 ml. H_2O 30 min.	30	—	NF	2·59	0·90	1·15	0·54
3. +10 ml. H_2O 30 min.; +2 ml. 10% glucose 20 min.	50	—	F	2·22	0·54	0·99	0·69
4. +10 ml. H_2O 30 min.; +2 ml. 10% glucose 60 min.	90	—	SF	2·46	0·70	1·12	0·64
5. +10 ml. H_2O 30 min.; +2 ml. 10% glucose 20 min. oxygenated throughout	50		—	2·10	0·43	0·91	0·76
Y. 10. Brewer's yeast grown in yeast extract in laboratory.							
6. +10 ml. H_2O 25 min.	25	—	NF	4·60	0·99	3·15	0·45
7. +10 ml. H_2O 25 min.	25	-	NF	4·60	0·94	3·26	0·45
8. +10 ml. H_2O 25 min.; +2 ml. 10% glucose 25 min	50	—	F	3·90	0·59	2·91	0·40
9. +10 ml. H_2O 25 min.; +2 ml. 10% glucose 25 min.	50	—	F	3·97	0·59	2·95	0·41
Y. 11. Similar to Y. 10.							
10. +10 ml. H_2O; not incubated	0	6	—	2·83	0·77	1·81	0·25
11. +10 ml. H_2O 15 min.	15	—	NF	3·48	0·87	2·32	0·29
12. +10 ml. H_2O 15 min.; +1 ml 10% glucose 15 min.	30	—	F	2·97	0·52	2·11	0·34
13. +10 ml. H_2O 15 min.; +1 ml. 10% glucose 15 min.	30	—	F	2·85	0·49	2·02	0·34
14. +10 ml. H_2O 15 min.; +1 ml. 10% glucose 35 min.	50	—	SF	3·12	0·78	2·06	0·28
Y. 12. Brewery press yeast.							
15. +10 ml. H_2O; not incubated	0	10	—	2·43	0·90	1·05	0·48
16. +10 ml. H_2O 15 min.	15	—	NF	2·91	1·02	1·32	0·57
17. +10 ml. H_2O 15 min.; +1 ml. H_2O 15 min.	30	—	NF	2·94	1·05	1·41	0·48
18. +10 ml. H_2O 15 min.; +1 ml. 10% glucose 15 min.	30	-	F	2·61	0·66	1·35	0·60
19. +10 ml. H_2O 15 min.; +1 ml. 10% glucose 30 min.	45	—	<F	2·88	0·75	1·53	0·60
20. +10 ml. H_2O 15 min.; +1 ml. 10% glucose 60 min.	75	—	SF	2·94	0·93	1·56	0·45
21. +10 ml. H_2O 15 min.; +1 ml. 10% glucose 60 min. +1 ml. glucose 15 min.	90	—	F	2·82	0·57	1·56	0·69
22. +10 ml. H_2O +1 ml. NaF 15 min.; +1 ml. H_2O 15 min	30	—	NF	2·94	0·57	1·35	1·02
23. +10 ml. H_2O +1 ml. NaF 15 min.; +1 ml. glucose 15 min.	30	—	Inhibited	3·21	0·42	1·65	1·14
24. +10 ml. H_2O +1 ml. IA 15 min.; +1 ml. H_2O 15 min.	30	—	NF	3·21	0·87	1·56	0·78
25. +10 ml. H_2O +1 ml. IA 15 min ; +1 ml. glucose 15 min.	30	—	Inhibited	3·21	0·90	1·50	0·81
26. +10 ml. H_2O +1 ml. toluene 5 min.	5	—	—	3·41	1·26	1·32	0·83
27. +10 ml. H_2O +1 ml. toluene 5 min.; +1 ml. H_2O 15 min.	20	—	NF	4·08	2·14	0·77	1·17
28. +10 ml. H_2O +1 ml. toluene 5 min.; +1 ml. glucose 15 min.	20	—	<F	3·93	2·09	0·71	1·13

Solutions: 10% glucose. 1 ml. = 25 ml. CO_2.
0·1 *M* sodium fluoride.
0·1 *M* sodium iodoacetate.
Rate of fermentation ml. CO_2 per 5 min. per g. yeast at 30°: Autofermentation <0·2
Maximum with sugar 5·0

Symbols: NF, no sugar added.
F, fermenting at maximum rate.
SF, sugar completely fermented.

is incubated with water alone, the experiments were so arranged that comparison could be made between samples incubated for equal times which (*a*) had received no sugar, (*b*) were fermenting sugar at the maximum rate and (*c*) had completely fermented the sugar added. The experiments are recorded in Table II and for convenience the differences in the various fractions between fermenting and non-fermenting samples are summarized in Table III.

The results show that during autofermentation of living yeast, the orthophosphate content is not appreciably changed but that there is some variation in the organic and labile phosphate contents. In yeast fermenting sugar the orthophosphate is decreased in amount (approximately 0·2–0·3 mg. P per g. yeast) but returns to the original level as fermentation ceases. This decrease persists in the presence of fluoride but not in that of iodoacetate, the fermentation being inhibited in each case. The slight increase in the organic phosphorus occasionally found in no case balances the decrease in orthophosphate as might be expected

Table II.

Treatment	Temp. ° C.	Total incubation time min.	% cells stained	Type of fermentation	Acid-soluble phosphorus mg. P per g. yeast Total P	Ortho-P	Labile P	Organic P
Y. 13. Brewery vat yeast.								
1. +12 ml. H_2O; not incubated	—	0	0	—	2·76	0·96	1·29	0·54
2. +11 ml. H_2O 15 min.	18	15		NF	2·67	0·96	1·20	0·51
3. +11 ml. H_2O 30 min.	,,	30	—	NF	2·46	0·96	1·05	0·48
4. +11 ml. H_2O 45 min.	,,	45	—	NF	2·76	0·96	1·29	0·51
5. +11 ml. H_2O 60 min.	,,	60	—	NF	2·76	0·99	1·20	0·57
6. +11 ml. H_2O 60 min.; +1 ml. 5% glucose 20 min.	,,	80	—	F	2·40	0·72	1·05	0·63
7. +10 ml. H_2O +1 ml. 5% glucose 10 min.	,,	10	-	F	2·91	0·66	1·59	0·66
8. +10 ml. H_2O +1 ml. 5% glucose 30 min.	,,	30	—	F	2·67	0·69	1·38	0·60
9. +10 ml. H_2O +1 ml. 5% glucose 60 min.	,,	60		<F	2·70	0·74	1·43	0·53
10. +10 ml. H_2O +1 ml. 5% glucose 60 min.; +1 ml. glucose 15 min.	,,	75	—	F	2·43	0·60	1·14	0·69
11. +11 ml. H_2O 15 min.	30	15	-	NF	3·12	0·90	1·65	0.57
12. +10 ml. H_2O +1 ml. 5% glucose 15 min.	,,	15	—	F	3·03	0·72	1·83	0·48
Y. 14. Brewery vat yeast.								
13. +12 ml. H_2O; not incubated	—	0	3·3	—	2·49	0·81	1·02	0·66
14. +11 ml. H_2O 15 min.	24	15	—	NF	2·22	0·78	0·84	0·60
15. +11 ml. H_2O 60 min.	,,	60	—	NF	2·28	0·81	0·84	0·63
16. +11 ml. H_2O 60 min.; +1 ml. 5% glucose 15 min.	,,	75	—	F	1·74	0·51	0·66	0·57
17. +11 ml. H_2O 120 min.	,,	120	4·5	NF	2·52	0·87	1·08	0·57
18. +11 ml. H_2O 120 min.; +1 ml. 5% glucose 15 min.	,,	135	—	F	2·31	0·63	0·96	0·72
19. +10 ml. H_2O +1 ml. 5% glucose 15 min.	,,	15	—	F	2·25	0·57	1·05	0·63
20. +10 ml. H_2O +1 ml. 5% glucose 60 min.	,,	60	—	SF	2·26	0·81	0·84	0·61
21. +10 ml. H_2O +1 ml. 5% glucose 120 min.	,,	120	--	SF	2·30	0·81	1·11	0·38
22. +10 ml. 5% glucose +1 ml. H_2O 120 min.	,,	120	6·6	F	2·70	0·39	1·56	0·75
23. +11 ml. H_2O, 24 hr. +1 ml. H_2O 15 min.	,,	24 hr.	—	NF	3·09	1·11	1·53	0·45
24. +11 ml. H_2O, 24 hr. +1 ml. glucose 15 min.	,,	24 hr.	—	F	3·10	0·87	1·68	0·55
25. +11 ml. H_2O 120 min.	37	120 min.	6·8	NF	3·27	1·17	1·41	0·69
26. +10 ml. 10% glucose +1 ml. H_2O 120 min.	,,	,, ,,	19·0	F	2·55	0·66	1·08	0·81

Symbols as in Table I.
Rates of fermentation. ml. CO_2 per 5 min. per g. yeast at 18°. Autofermentation 0. Maximum with sugar 1·5.
Rates of fermentation. ml. CO_2 per 5 min. per g. yeast at 24°. Autofermentation <0·1. Maximum with sugar 1·9.

Table III. *Difference in acid-soluble phosphate content between fermenting and non-fermenting yeast (from Table II, F–NF).*

Yeast	Temp. ° C.	Incubation time	Acid-soluble mg. P per g. yeast Total	Ortho-P	Labile P	Organic P
Y 13	18	10–15 min.	+0·24	-0·30	-0·39	+0·15
		30 ,,	+0·21	-0·27	+0·33	+0·12
		60–80 ,,	-0·33	-0·39	-0·06	+0·12
			-0·36	-0·29	-0·15	+0·06
Y 14	24	15 ,,	+0·03	-0·21	+0·21	+0·03
		60–75 ,,	-0·54	-0·30	+0·18	-0·06
		120–135 ,,	-0·21	-0·24	-0·12	+0·15
		24 hours	+0·01	-0·24	+0·15	+0·10
Y 13	30	15 min.	-0·09	-0·18	+0·18	-0·09
Y 14	37	120 ,,	-0·72	-0·51	-0·33	+0·12

if hexosephosphoric esters were formed. Taking into account the limited accuracy of determination of the labile and organic phosphate values, and the changes during autofermentation, the variation in these fractions could not be consistently related to the onset of fermentation. Lohmann [1928, 2] showed that the amount of phosphate extracted by trichloroacetic acid from baker's yeast was increased by preliminary heating of the yeast in water, or by addition of toluene, and that much of this phosphate was acid-labile. This is true also of the brewery yeast used in these experiments (Table IV). The variation in labile and

Table IV. *Increase in acid-soluble P after preliminary treatment of yeast.*

Treatment	mg. acid-soluble P per g. yeast Total P	 Ortho-P	 Pyro-P
(1) 1 g. + 10 ml. H_2O + 3 ml. 25 % trichloroacetic acid	2·91	0·87	1·59
(2) 1 g. + 10 ml. H_2O 2·25 min. at 100°, cooled + trichloroacetic acid	3·84	1·02	2·13
(3) 1 g. + 10 ml. N HCl 7 min. at 100°, cooled + trichloroacetic acid	6·35	4·50	—

Total P by ashing 6·35 mg. per g. yeast.

organic phosphate may therefore be due to a shift between acid-soluble and insoluble phosphorus compounds, and the disappearance of orthophosphate may be ascribed as justly to the formation of an acid-insoluble as to that of an acid-soluble compound, especially as in many cases the decrease coincided with a decrease in the total acid-soluble phosphate.

Phosphorus compounds of yeast.

Pressed yeast contains about 6·0 mg. P per g., of which 50 % can be extracted with trichloroacetic acid, approximately 15 % as orthophosphate, 25 % as a labile phosphoric acid shown by Lohmann [1928, 1] to contain inorganic pyrophosphate and 10 % in organic combination. The latter probably includes cozymase and Warburg's coferment (about 1 %) as well as adenosinetriphosphate (2 %) and adenylic acid. Calculated from the yields which have been obtained 8–12 % of the phosphorus is present in (acid-insoluble) nucleic acid [see Levene & Bass, 1931] and 2 % as lipin-P [MacLean & Daubney, 1927], whilst from the flavin content [Pett, 1935] 0·05 % may be present as flavinmonophosphate. The phosphorus compounds enumerated account for 65 % of the total phosphorus of yeast: before attempting to identify the unknown compound formed by the orthophosphate disappearing during fermentation, it was necessary to obtain a very precise idea of the nature of the remaining phosphorus.

Extraction of phosphorus compounds from the yeast cell.

Yeast obtained from the brewery two days after pitching was washed 3 times with water, filtered and pressed to a cake on Büchner funnels. The cake was then submitted in succession to the following treatments.

Extract 1. The yeast was well mixed with 5 % trichloroacetic acid (300 ml. per 100 g. yeast), filtered and re-extracted with half the original volume of acid, the filtrates being combined.

Extract 2. The residual yeast cake was suspended in five parts of water (pH about 2·0) and sufficient 2 N NaOH (about 15–20 ml. per 100 g. yeast) added to bring the pH to 8·6–9·0. As the sodium hydroxide was added, part of the cell contents passed rapidly into the solution. The latter, which was reddish brown in colour, filtered fairly easily through a No. 3 (Whatman) paper under suction; and on addition of two volumes methcol gave a flocculent precipitate containing >90 % of the phosphorus present in the extract; yield, after washing with alcohol and drying, about 1·0 g. per 100 g. yeast.

Extract 3. The yeast cake was again suspended in water and re-filtered giving a clear yellow filtrate from which the phosphorus compounds present were precipitated by addition of methcol either as sodium or barium salts.

At this stage the yeast cell membrane was still unruptured. An attempt was made with one batch of yeast to extract the remaining phosphorus by grinding

Table V.

Yeast no.	Preliminary treatment	% total P of yeast found in									Analyses of Na salts precipitated by methcol from extract 2							
		Extract 1. trichloroacetic			Extract 2 aq. neutral			Extract 3 2nd aqueous			% of total P of yeast in salt	% composition					$[\alpha]_{5461}$	% P hydrolysed in 7 min.
		Total P	Ortho-P	Labile P	Total P	Ortho-P	Labile P	Total P	Ortho-P	Labile P		C	H	N	P	Na		
16	None	61	17	33	23	0	19·4	7·8	0	4·7	20·6	—	—	9·3	16·0	10·3	+54°	83
17	None	52	—	—	24	0	22	8·9	0	—	19·0	—	—	7·1	18·6	—	+27	92
18	None	39	14	—	36	0	32	7·2	0	—	33·0	—	—	5·0	17·9	—	+36	89
21 A	None	45	17	21	28	0	26	4·4	0	—	—	—	—	—	—	—	—	—
22 A	None	45	18	22	31	0	28	9·0	0	—	29	—	—	6·9	16·0	—	+32	85
14 C	None	45	13	19	36	0	28	—	—	—	36	Precipitated as very insoluble Ba salts						
14 B	Autofermentation + toluene + NaF	68	19	3	25	0	15	—	—	—	12	Precipitated as very insoluble Ba salts						
15 C	Autofermentation, no toluene, 4 hr.	37	16	17	32	0	25	8·4	—	—	25	19·9	3·4	8·6	16·3	10·3	+43	80·5
15 A	Autofermentation, + toluene, 4 hr.	62	46	2	18	0	8	0	—	—	11	30·1	4·1	13·5	8·5	6·9	+73	43·0
	Theory for tetrasodium nucleate										—	32·8	3·6	15·1	8·9	6·6	$[\alpha]_D$ +60	—

the yeast cake (200 g.) with equal parts of sand and kieselguhr. The ground mass was then extracted successively with 1 % saline (extract 4), 5 % trichloroacetic acid (extract 5) and water + NaOH at *p*H 9·0 (extract 6), which removed 1·8, 1·5 and 1·5 % of the total phosphorus respectively, together with 2 g. glycogen (extract 4) and 1 g. protein (extract 6). The phosphorus contained in the last four extracts, though small in amount, may be qualitatively important; up to the present attention has been paid chiefly to the phosphorus compounds present in the first two extracts. The distribution in different batches of yeast is shown in Table V.

Phosphorus compounds present in the trichloroacetic acid extract.

Extract 1. The trichloroacetic acid filtrate was treated with a slight excess of barium acetate, the *p*H adjusted to 8·0 with baryta and 1/10 volume alcohol added. The precipitate of sparingly soluble barium salts was removed by filtering. After estimation of the phosphorus present the filtrate was treated with basic lead acetate in slight excess, any precipitate removed and the phosphorus remaining in solution estimated. It has been generally assumed that hexosediphosphate, which forms a sparingly soluble barium salt, and hexosemonophosphate, which forms a soluble barium and a sparingly soluble basic lead salt, are present in fresh yeast, though no rigorous identification has been made. The percentage of the total phosphorus of yeast allotted to the hexosediphosphate and hexosemonophosphate fractions (Table VI) on the basis of this separation shows that the concentration of these esters, if they are present at all, is very small [see Boyland, 1930], but some of the phosphate left in solution after treatment with basic lead acetate may be hexosemonophosphate.

Table VI. *Distribution of acid-soluble phosphorus in fresh yeast.*

	% of total P of yeast.					
Yeast	Total acid-sol.	Organic P	P in 10 % alcohol filtrate	P in basic Pb filtrate	P as "hexose diphosphate"	P as "hexose monophosphate"
1	46	7	4·7	4·2	2·3	0·5
2	41	8	6·7	4·8	1·3	1·9
3	39	6·3	4·7	—	1·6	—
13	49	10·1	5·7	5·2	4·4	0·5

As a consequence of the identification of metaphosphoric acid in the acid-insoluble portion of yeast, described in the next section, the sparingly soluble barium salts from the acid extract were tested for the presence of metaphosphate with positive results.

Trichloroacetic acid filtrate from 200 g. fresh yeast (Y 20). Total P 495 mg.; ortho-P 89 mg.; labile P 289 mg. 5 g. barium acetate and 0·25 volume alcohol were added (*p*H 2) giving 1·2 g. barium precipitate (P, 16·7 %; 200 mg. P). 1·17 g. of this salt were extracted three times with 10 ml. H_2O.

Analysis of water-insoluble portion. Wt. 0·94 g. 173 mg. P (= 60 % recovery of original labile P). P, 18·4 %, of which 96 % was hydrolysed in 7 min. in *N* HCl at 100°. Ba, 41·9 %; N, 0·4 %. Atomic ratio P/Ba = 2·0/1·03. 1·6 mg. of this salt in acid solution gave a white precipitate with 1 ml. of peptone solution, indicating metaphosphoric acid. The analyses of barium metaphosphate and acid barium pyrophosphate are very similar; ($Ba(PO_3)_2$ requires P, 20·99 %; Ba, 46·51 %; $BaH_2P_2O_7$; P, 19·8 %; Ba, 43·7 %). An attempt was made to prepare the acid barium salt of pyrophosphoric acid; after three reprecipitations with alcohol at *p*H 2·0 of a HCl solution of barium pyrophosphate, the precipitate obtained was partly soluble in water and was obviously still a mixture of the neutral and acid salts (P, 16·4 %; Ba, 45·8 %; ratio P/Ba = 2/1·25).

It was concluded that part of the acid-soluble labile phosphoric acid of yeast is present as metaphosphate; it may be recalled that Lohmann [1928, 1] remarked upon the colloidal nature of the labile phosphoric acid fraction in yeast, though he succeeded in isolating sodium pyrophosphate in crystalline form.

Isolation of a labile phosphoric acid compound from the acid-insoluble portion of the yeast cell.

Extract 2, aqueous extract at *p*H 9·0. The analyses of the sodium salts precipitated by addition of methcol to this extract from different batches of yeast are given in Table V. The salts were light brown in colour, very soluble in water and had a high surface tension. The re-dissolved substance filtered very badly and frequently was not re-precipitated by alcohol at *p*H 8·0, though acidification to *p*H 6·0–7·0 gave immediate flocculation. On acidifying with glacial acetic acid to *p*H 3·0 the aqueous solution (10 %) became opalescent but did not flocculate. All the salts contained iron (1–2 %) and gave a blue colour on boiling with orcinol in 50 % HCl.

The salts obtained from fresh yeast, either resting or after autofermentation, contained 16–18 % P (of which 80–90 % was hydrolysed in 7 min. in *N* HCl at 100°) and approximately 7 % N, the atomic ratio P/N being of the order 12/14. The salt obtained in a similar manner from yeast which had been allowed to autoferment after addition of toluene (Y15A) contained only 8·5 % P (of which 43 % was labile), the P/N ratio being 4/14. The analysis of this salt corresponds closely with that required for the tetrasodium salt of nucleic acid; it has been assumed for the present, without identification of individual nucleotides, that the nitrogenous component of these labile phosphoric compounds is essentially ribonucleic acid on the following grounds:

(*a*) The percentage composition and molecular rotation.

(*b*) The physical properties.

(*c*) The absolute yield (corresponding to about 10 % of the total P) which agrees with the yields of nucleic acid previously obtained.

(*d*) Failure to find the required amount of nucleic acid in other fractions.

It is of course possible that the salts are merely mixtures of a nucleate and a labile inorganic phosphate; but, since the latter might be expected to be soluble in trichloroacetic acid, it was tentatively concluded that the substance containing nucleic acid and labile phosphoric acid was present as a compound in living yeast and could be enzymically dephosphorylated in the presence of toluene with the ultimate production of orthophosphate and nucleic acid.

Identification of metaphosphoric acid as a component of the nucleic acid complex.

Fractionation with HCl and alcohol. The sodium salt (8·5 g. containing 1320 mg. P of which 1160 mg. were labile) from extract 2 (Y 16 and Y 17) was dissolved in 100 ml. H_2O and 3 ml. conc. HCl were added. A small flocculent precipitate formed, but the bulk of the substance remained in a very fine suspension which did not deposit on centrifuging. The supernatant fluid was decanted and treated with 3 volumes methcol (*p*H 2·0) giving a granular precipitate. The latter was redissolved 5 times in 10 parts of 5 % HCl and reprecipitated with alcohol, each time removing by centrifuging a small acid-insoluble portion, until finally the solution was only faintly opalescent. The precipitate at this stage was white and almost entirely inorganic in nature. (Wt. 2·66 g. (mg. P 670) P, 25·1 %; Na, 8·2 %; Fe approx. 1 %; N < 1·0 %. Atomic ratio P/Na = 2·2/1.) The analysis was unchanged after two more extractions.

For identification the following salts were prepared from the acid sodium salt:

(*a*) Neutral sodium salt. 1·06 g. were dissolved in 10 ml. water (*p*H 2·0) and one equivalent of NaOH added (*p*H 10). The solution failed to crystallize and the neutral salt (1·15 g.) was precipitated with alcohol. It was taken up in 8 ml. water and dissolved on warming to 40°, but suddenly formed a gel on filtering.

(*b*) Neutral barium salt. 0·43 g. acid sodium salt (109 mg. P) was dissolved in 10 ml. water and the *p*H adjusted to 7·0 with a few drops of cold baryta. 1·0 g. barium acetate in solution was added and the resulting white precipitate filtered, washed with water and dried; wt. 0·54 g.; 102 mg. P (= 93 % recovery). Analysis, P, 19·7 %: orthophosphate nil; 100 % hydrolysis in 7 min. in *N* HCl at 100°, Ba, 43·7 %; N nil; Fe approximately 0·8 %; atomic ratio P/Ba = 1·99/1. ($Ba(PO_3)_2$ requires P, 20·99 %; Ba, 46·51 %. The salt was very insoluble in water and tended to form a colloidal solution in dilute HCl, from which the barium was not precipitated by dilute H_2SO_4 in the cold. 1·0 ml. of the free acid, prepared by decomposition with the theoretical amount of H_2SO_4, containing 0·1 mg. P, gave a white precipitate with 1·0 ml. 2 % peptone solution, indicating metaphosphoric acid. No precipitate was obtained by addition of H_2SO_4, HCl, $BaCl_2$ or sodium pyrophosphate + acetic acid to a peptone solution.

(*c*) Neutral silver salt. Some difficulty was at first met in preparing this salt from dilute solutions, owing to its colloidal nature. 0·2 g. acid sodium salt (50 mg. P) was dissolved in 5 ml. water + NaOH to *p*H 7·0 and 5 ml. 10 % $AgNO_3$ were added, giving a white precipitate. This was separated and washed twice with water at the centrifuge, the supernatant liquid being very opalescent, and dried in a vacuum desiccator. The yield was poor (66 % recovery) and the salt gave a slightly brown solution; wt. 0·22 g.; P, 15 %; Ag, 53·1 %: Ag/P 1·02/1. For comparison a sample of silver pyrophosphate was prepared without difficulty giving on analysis P. 10·7 %; Ag, 66·5 %, ratio Ag/P 1·8/1. (Theory for $Ag_4P_2O_7$ P, 10·2 %; Ag, 70·7 %; for $AgPO_3$ P, 16·5 %: Ag, 57·7 %.)

There seems no reasonable doubt from the analyses and properties of these salts that at least 50 % of the labile phosphoric acid in the nucleic acid complex is metaphosphoric acid.

The various acid-insoluble precipitates from the HCl extraction were collected, dissolved in dilute NaOH and reprecipitated with methcol. The salts were rather more soluble in alcohol than previously and still contained a high proportion of labile P. Analysis, P, 12·3 %; N, 5·9 %; wt. 2·10 g. It is possible that prolonged extraction with 5 % HCl may split the nucleosidic linkage; only 28 % of the nitrogen was recovered in the acid-insoluble fractions, the remaining phosphorus and nitrogen being found in the alcohol filtrates.

In recent years the occurrence of metaphosphoric acid in organic material has been so rarely mentioned that it was thought that this nucleic acid-metaphosphoric acid complex had not previously been described. In fact Kossel [1893] isolated from yeast an acid "Plasminsäure" which he thought was a metaphosphoric acid closely associated within the cell with nucleic acid. Ascoli [1899], a pupil of Kossel, purified the "Plasminsäure" by extraction in hydrochloric acid, prepared the silver and strychine salts[1] and again concluded from the analytical reactions that the acid was a metaphosphoric derivative. There can be no doubt that the nucleic acid complex now described is Kossel's Plasminsäure.

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[1] The salts all contained iron. Ascoli remarks the fact that both meta- and pyro-phosphoric acids inhibit the usual colour reactions of ferric iron.

DISCUSSION.

The fact that the process of fermentation of sugar by living yeast coincides with a decrease in the orthophosphate content of the cells affords strong evidence that phosphorylation is concerned in the glycolysis. The fate of the esterified phosphate is unknown, but the effects of fluoride and iodacetate on the phosphorylation indicate a general resemblance to this process in yeast juice.

The hypothesis that phosphorylation of carbohydrate is obligatory in glycolysis by all living cells is supported by the widespread distribution of phosphorus compounds known to intervene as coenzymes in cell-free glycolysis; by the phosphorylation of carbohydrate during glycolysis in dead or cell-free preparations not only of yeast, muscle and bacteria but also of higher plants [see Tankó, 1936]; and by the necessity for phosphate-carrying coenzymes in lactic acid formation by extracts of animal tissues [Euler *et al.* 1936; Boyland & Boyland, 1935]. A direct comparison between the glycolytic processes of cells and cell-free extracts may be misleading because of the different conditions, e.g. accessibility of substrate, presence of coenzyme, existing in these systems. The view that enzymic glycolysis can take place without phosphorylation is based chiefly on such a comparison [Ashford & Holmes, 1929; Bumm & Fehrenbach, 1931; Ashford, 1933; 1934]. Phosphorylation is not necessarily demonstrable by an accumulation of hexosephosphoric esters; whilst a stoichiometric relation between esterification and decomposition of hexose appears to be peculiar to yeast preparations. Nord *et al.* [1936] are also of the opinion that phosphorylation may be unnecessary, but they do not take into account the possibility that during glycolysis by living cells a change in the phosphorus distribution may be internal and not external to the cell. During glycolysis in the yeast cell the fluctuations in the acid-soluble phosphorus balance are of a small order (5 % of the total phosphorus) yet the rate of glycolysis in yeast is comparatively high. It seems possible therefore that a change in the phosphorus distribution during glycolysis in organisms or tissues less favourable in this respect may be so small in amount as to lie within the experimental error, especially if inorganic phosphate is added to the medium.

The occurrence of metaphosphoric acid in close association with nucleic acid and iron in the yeast cell arouses interest in the physical and chemical roles of this acid in the cell metabolism. The ease with which the nucleic acid complex was obtained free from extraneous material is probably due to the resistant nature of the yeast cell membrane whose continuity remains, even if its permeability has been altered, after treatment with trichloroacetic acid. It seems probable that the latter precipitates the nucleic acid complex as a nucleoprotein and that on subsequent neutralization the nucleic acid passes into solution through the cell wall whilst any redissolved protein, glycogen etc. is held back. A single attempt to obtain protein-free nucleic acid from pancreas by this method was unsuccessful; but the technique could probably be applied to the isolation of nucleic acid from yeast or other cells with a cellulosic membrane.

Since maceration extract [Lohmann, 1928, 2] and yeast juice contain only small amounts of "labile" phosphate, it is possible that the presence of this nucleic acid-metaphosphoric acid complex is not obligatory in enzymic glycolysis. It may however prove to be of importance in the economy of cell glycolysis. The modified fermentation brought about by the addition of toluene to the yeast cell is accompanied by decomposition of the nucleic acid complex with formation of orthophosphoric acid [see also Lewitow, 1936]; whilst the intact cell may be incubated in the absence of sugar without appreciable change. Preliminary experiments indicate that during the fermentation of sugar the composition of the nucleic acid fraction is significantly altered; further experiments are now in progress.

SUMMARY.

1. At the onset of fermentation of sugar by fresh brewery yeast the acid-soluble orthophosphate content of the cells decreases in amount to a fairly constant value and regains the original level when fermentation ceases. This fact is evidence that phosphorylation is concerned in the glycolytic process of the yeast cell.

2. The coincident changes in total, labile and organic (acid-soluble) phosphate could not be consistently related to the fermentation process. Nothing is known of the nature of the compound formed by the disappearing orthophosphate, but the phosphorus balance suggests that it may be acid-insoluble.

3. A process is described by which different phosphorus compounds can be successively extracted from fresh yeast.

4. Metaphosphoric acid has been shown to be present in the trichloroacetic acid extract of fresh yeast, but neither hexosedi- nor hexosemono-phosphoric ester has yet been identified.

5. Approximately 30% of the total phosphorus of yeast is present as a complex containing iron, nucleic acid and metaphosphoric acid. By extraction of the nucleic acid complex with hydrochloric acid, inorganic metaphosphate was obtained and identified. The nucleic acid complex is probably identical with the "Plasminsäure" described by Kossel.

6. During the autofermentation of yeast in the presence of toluene the metaphosphoric acid present in the nucleic acid fraction was decomposed, with the ultimate production of orthophosphate.

I wish to record my gratitude to Prof. R. Robison for his invaluable advice and criticism.

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CXCVI. THE SYNTHESIS OF URIC ACID IN THE AVIAN ORGANISM: HYPOXANTHINE AS AN INTERMEDIARY METABOLITE.

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It has been demonstrated that the co-operation of two organs, liver and kidney, is necessary for uric acid synthesis in pigeons [Krebs & Benzinger, 1933; Schuler & Reindel, 1933, 1, 2]. On this account it is possible to resolve the complex process into two parts which may be studied individually. In this paper it will be shown that the primary process is a ring synthesis, occurring in the liver and yielding hypoxanthine, which is then oxidised to uric acid by the kidney (or by certain other tissues) under the influence of xanthine oxidase. Thus in pigeons xanthine oxidase is a component of the system which synthesizes uric acid.

METHODS.

The tissue slice technique was employed, the medium being the physiological saline originally devised for mammalian tissues [Krebs & Henseleit, 1932]. This procedure is permissible, because the ionic composition of bird's serum does not differ significantly from that of mammalian serum [Dyer & Roe, 1934; Morgan & Chichester, 1935]. The concentration of bicarbonate was 0·025 *M* and the saline was in equilibrium with an atmosphere of $5\% \ CO_2 + 95\% \ O_2$. The *p*H was 7·4. Substrates were added in neutral solution: acids as sodium salts, ammonia as chloride.

The slices were prepared in the usual way and washed with saline as described by Krebs & Henseleit [1932]. The temperature of the thermostat was 40° unless otherwise stated. The tissue (10–20 mg. dry weight) was immersed in 3·0 ml. saline and shaken usually for 2 hours, after which the change in concentration of metabolites was sufficient for accurate determination.

Uric acid was estimated manometrically [Edson & Krebs, 1936].

Quantities of metabolites are expressed as μl. gas in the customary way. 17 mg. ammonia are considered equivalent to 22,400 μl. and 1 millimol uric acid (168 mg.) is made equivalent to $2 \times 22{,}400$ μl. CO_2, since 1 molecule uric acid yields 2 molecules CO_2 in the determination.

The rate of metabolism is expressed by the following quotients:

$$Q_{NH_3} = \frac{\mu\text{l. } NH_3}{\text{mg. tissue (dry weight)} \times \text{hours}},$$

$$Q_{\text{uric acid}} = \frac{\mu\text{l. } CO_2}{\text{mg. tissue (dry weight)} \times \text{hours}}.$$

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The site of uric acid formation in pigeons.

It is known that pigeon liver and kidney slices form mere traces of uric acid when the tissues are examined separately, but considerable quantities are produced if both tissues are present together in ammonia-containing saline [Krebs & Benzinger, 1933; Schuler & Reindel, 1933, 1]. Schuler & Reindel [1935] published experiments, moreover, which suggest that pancreas slices may replace kidney. Experiments in which uric acid was determined manometrically are in agreement with these previous observations (Table I).

Table I. *Uric acid production by pigeon tissues.*

Tissue present (mg.)	Uric acid formed in 2 hrs. µl. CO_2
1. Liver (21·03)	9·1
2. Kidney (11·59)	10·6
3. Pancreas (14·99)	13·4
4. Liver (13·67) + kidney (15·89)	56·6
5. Liver (19·24) + pancreas (5·92)	35·0

The disappearance of ammonia.

In attempting to elucidate the separate functions of the relevant tissues we first identified the tissue that binds ammonia. Slices of the different tissues were shaken alone in saline containing ammonium chloride, and after 1 or 2 hours

Table II. *Disappearance of ammonia in the presence of bird's liver slices.*

Liver mg.	State of nutrition	Substrate	Amount of NH_3 per flask µl. Initial	Amount of NH_3 per flask µl. Final	Duration of exp.	NH_3 used µl.	$-Q_{NH_3}$
		I. Pigeon.					
15·66	Starved 48 hrs.	Nil	406	314	2 hrs.	92	2·93
13·47		*dl*-Lactate, 0·02 *M*	,,	157		249	9·24
13·48		Pyruvate, 0·02 *M*	,,	104		302	11·2
13·01		Glycerol, 0·02 *M*	,,	294		112	4·30
13·37		α-Ketoglutarate, 0·02 *M*	,,	270		136	5·09
		II. Pigeon.					
28·94	Starved 24 hrs.	Nil	390	135	2 hrs.	255	4·41
22·75		Succinate, 0·02 *M*	,,	175		215	4·72
24·38		α-Glycerophosphate, 0·02 *M*	,,	262		128	2·63
19·49		Pyruvate, 0·02 *M*	,,	26		364	9·34
24·13		Acetate, 0·02 *M*	,,	295		95	1·97
20·53		*l*(+)-Glutamate, 0·02 *M*	,,	207		183	4·46
25·90		Glucose, 0·02 *M*	,,	149		241	4·65
28·50		Glycine, 0·02 *M*	,,	168		222	3·90
23·92		Glycerol, 0·02 *M*	,,	81		309	6·46
22·72		*n*-Octanoate, 0·02 *M*	,,	293		97	2·13
29·98		Ornithine, 0·02 *M*	,,	142		248	4·14
		III. Pigeon.					
8·69	Well fed	Nil	448	240	2 hrs.	208	12·0
7·16		*dl*-Lactate, 0·018 *M*	,,	213		235	16·5
		IV. Pigeon.					
19·23	Well fed	Nil	515	278	100 min.	237	7·40
14·95		Pyruvate, 0·01 *M*	,,	156		359	14·2
		V. Fowl.					
16·61	Well fed	Nil	416	182	2 hrs.	234	7·04
21·29		*dl*-Lactate, 0·01 *M*	,,	140		276	6·49
20·50		Pyruvate, 0·01 *M*	,,	57		359	8·75
19·90		β-Phosphoglycerate, 0·01 *M*	,,	172		244	6·14
21·19		α-Ketoglutarate, 0·01 *M*	,,	105		311	7·07
19·73		α-Glycerophosphate, 0·01 *M*	,,	110		306	7·75

ammonia was determined by the method of Parnas. Neither kidney nor pancreas removed added ammonia, whereas liver caused the disappearance of large amounts. Table II shows the rate of ammonia disappearance in pigeon's and fowl's liver in the presence of various substrates. We added these substances expecting to obtain information about the source of the carbon skeleton of uric acid. Although lactic and pyruvic acids have a more marked effect than other substrates especially in the starved animal, it seems premature in view of the complex nature of the systems involved to draw definite conclusions from this fact.

The effect of xanthine oxidase prepared from milk.

The traces of uric acid formed by pigeon's liver account for about 5 % of the ammonia which has disappeared. If, however, the total nitrogen in solution is determined by Kjeldahl's procedure, no significant decrease is observed during ammonia disappearance. This shows that the nitrogenous substance which is formed from ammonia, and which is not uric acid, is returned to the solution by the tissue slices, and the problem of its identity arises.

It is characteristic of this substance that it yields uric acid with kidney and pancreas of the pigeon. The work of Morgan [1926] suggested to us that the substance in question might be xanthine or hypoxanthine, because Morgan gave the following distribution of the enzyme in birds:

		Xanthine oxidase
Pigeon	Liver	–
	Pancreas	+
	Kidney	+
Fowl	Liver	+

We have confirmed these observations, using a more sensitive technique recently developed by Booth [1935].

The findings of Morgan are therefore in agreement with the view that the action of xanthine oxidase is the final step in uric acid synthesis in birds, which requires a second tissue in the pigeon but not in the fowl. In order to test this assumption we attempted to replace kidney and pancreas by a xanthine oxidase preparation ("whey preparation") made from milk according to the directions of Dixon & Kodama [1926]. The results in Table III show that the assumption is correct: pigeon's liver produces a substance which gives uric acid in presence of xanthine oxidase.

Table III. *Effect of milk xanthine oxidase on the pigeon's uric acid precursor.*

Tissue and enzyme	Uric acid formed in 2 hrs. μl. CO_2
1. Liver (21·03 mg.)	9·1
2. Xanthine oxidase (50 mg.)	3
3. Liver (23·02 mg.) + xanthine oxidase (50 mg.)	63

In view of the high specificity of xanthine oxidase it is probable that the uric acid precursor is either xanthine or hypoxanthine. The following measurement of the relation between oxygen uptake and uric acid formation shows that the substance behaves like hypoxanthine.

Pigeon liver slices (303 mg.) were shaken for 2 hours in ammonia-containing saline (50 ml.), and after removal of the slices bicarbonate was decomposed with hydrochloric acid, and phosphate buffer (*p*H 7·4) was added. 3·0 ml. of this solution were measured into a manometric cup and 0·1 ml. xanthine oxidase solution was placed in the side-bulb. The flask was equilibrated in the thermostat and the oxygen uptake was measured after addition of xanthine oxidase. When the oxygen uptake ceased uric acid was determined (Table IV).

Table IV. *Oxygen uptake and uric acid formation under the influence of xanthine oxidase.*

3·0 ml. Ringer solution in which pigeon liver slices had been shaken for 2 hrs. + xanthine oxidase.

Time min.	Oxygen uptake μl. O_2	Uric acid μl. CO_2
10	36·1	–
20	44·2	–
30	45·5	–
40	45·5	87·0

(1 mM uric acid = 2 mM CO_2)

If xanthine were the only substrate undergoing oxidation to uric acid, the molecular ratio O_2 consumed/uric acid formed would have the value 0·5, or if it were hypoxanthine, 1·0. The observed value is 45·5/43·5 = 1·04 which suggests that the purine base is hypoxanthine rather than xanthine. It should be mentioned that added xanthine and hypoxanthine yield the theoretical ratios under the conditions of the experiment recorded in Table IV.

The presence of the purine base is easily demonstrated by the methylene blue technique. Ringer solution containing ammonium salt in which pigeon liver slices have been shaken will reduce methylene blue rapidly if xanthine oxidase be added. 5·0 ml. of the solution mentioned above (303 mg. liver in 50 ml. saline) were mixed in an evacuated Thunberg tube with 0·2 ml. methylene blue solution (0·1 %) and 0·3 ml. 20 % xanthine oxidase suspension at pH 8·0. Reduction was complete in 3½ min. at 40°. A control without xanthine oxidase showed no reduction in 24 hours.

The isolation of hypoxanthine.

The above conclusions have been confirmed by the isolation and identification of hypoxanthine. The liver of a pigeon was cut into slices which were evenly distributed amongst three large shaking vessels, each of which contained 40 ml. of bicarbonate-Ringer solution. The Ringer solution was 0·02 M with respect to pyruvate and 0·007 M with respect to ammonium chloride. The slices were shaken for 2 hr. at 40°, the gas space containing a mixture of 5 % CO_2 and 95 % O_2.

After removal of the slices the fluid was deproteinized with 0·1 volume of 30 % trichloroacetic acid, and the purine base was separated by the method of Salkowski [1898]. The protein-free filtrate was treated with 20 ml. strong ammonia in order to remove phosphates. Since the calcium and magnesium ions present were more than equivalent to the phosphate ions the use of magnesia mixture was unnecessary. The clear filtrate (after phosphate precipitation) was reduced to one-eighth its original volume by evaporation on the water-bath. Purine-containing extracts from the livers of six pigeons were treated in the same way and combined, made strongly alkaline with ammonia and the purine precipitated with 10 ml. 5 % silver nitrate solution. The yellowish, gelatinous flocculum of silver purine was separated by centrifuging, washed twice with dilute ammonia and finally with distilled water. Previous tests had shown the absence of uric acid from the extract, and therefore none of the usual precautions for the separation of that substance was necessary.

The purine silver compound was dissolved in hot nitric acid (sp. gr. 1·1), from which it crystallized in small white needles immediately on cooling. This behaviour is characteristic of hypoxanthine silver nitrate. The product was recrystallized three times from hot nitric acid of sp. gr. 1·1. From 3·86 g. of liver (dry weight) 43 mg. hypoxanthine silver nitrate were obtained. In other experiments the yields were from 4·51 g. liver 55 mg. and from 5·60 g. liver 74 mg. hypoxanthine silver nitrate.

Free hypoxanthine was prepared by decomposing the silver nitrate compound with hydrogen sulphide. 30 mg. hypoxanthine silver nitrate were suspended in 20 ml. distilled water and the liquid saturated with H_2S. The filtrate from the silver sulphide was warmed and aerated to remove H_2S; it was then made weakly alkaline with ammonia and aerated again until it was free from ammonia and was then evaporated on the water-bath until the volume was 2 ml. When the solution was cooled in the ice-box, a finely divided, white substance was deposited: yield, 10 mg. This was recrystallized three times from distilled water and dried *in vacuo* for several days before analysis.

Elementary analysis (Dr Weiler, Oxford)

	C	H	N (Dumas)
Substance isolated	44·70	3·25	39·86
Substance isolated	—	—	39·48
Calculated for hypoxanthine	44·10	2·96	41·17
Calculated for xanthine	39·46	2·65	36·84
Pure hypoxanthine (Hoffmann-La Roche)	44·87	3·43	40·31
Pure hypoxanthine (Hoffmann-La Roche)	—	—	39·11

The values for the nitrogen (Dumas) tend to be low, but pure hypoxanthine gives the same low figures and the agreement between the figures obtained for hypoxanthine and for the substances isolated from liver is very satisfactory.

Properties of the substance isolated.

1. A white, micro-crystalline, non-deliquescent powder, sparingly soluble in cold water but rather more soluble in hot water.
2. The solution reduced methylene blue anaerobically in presence of xanthine oxidase.
3. When a solution of the substance was shaken with xanthine oxidase in air, it absorbed oxygen and formed uric acid.
4. It formed a salt with nitric acid and silver nitrate. When this salt was treated according to Strecker [1858], it gave a compound with the properties of hypoxanthine silver oxide; N, 15·96 %, the theoretical value being 15·63 % [Bruhns, 1890].

The above facts establish the identity of the uric acid precursor as hypoxanthine. This is the purine which Schuler and Reindel [1935] have recently stated to be formed by pigeon's liver.

The influence of cell structure.

There is no disappearance of ammonia in preparations of liver, the cell structure of which has been destroyed by processes such as grinding with sand.

It has been mentioned that kidney slices can be replaced effectively by xanthine oxidase prepared from milk. Further, a kidney preparation made by the method of Booth [1935] is an adequate substitute for slices, although cell structure is destroyed.

The quantitative relationship between ammonia disappearance and hypoxanthine formation.

Determinations of hypoxanthine show that the amount of hypoxanthine formed accounts for not more than 30% of the ammonia disappearing in the presence of lactate and pyruvate. There are other ammonia-binding mechanisms in liver and large quantities of amino-nitrogen are formed under some conditions, especially in the presence of lactate or pyruvate. These data and the methods employed will be published in a later communication.

Uric acid synthesis in other birds.

The presence of hypoxanthine could not be detected in saline in which fowl's or duck's liver slices had been shaken, but this does not contradict the hypothesis that hypoxanthine may also be the direct precursor of uric acid in these birds as well as in the pigeon, since the xanthine oxidase activity of hen's and duck's liver is so high that hypoxanthine would be removed more quickly than it was formed by synthesis. The experiments of Table V indicate that this is the case.

Table V. *Rates of uric acid formation from ammonium chloride and from hypoxanthine in fowl and duck livers.*

Animal	Substrate	$Q_{\text{uric acid}}$
1. Fowl	NH_4Cl; 0·01 *M* pyruvate	1·15
	Hypoxanthine 0·0025 *M*	1·97
2. Duck	NH_4Cl; 0·01 *M* *dl*-lactate	0·96
	Hypoxanthine 0·0025 *M*	4·76

SUMMARY.

1. In pigeons two tissues are necessary for uric acid synthesis. The primary step, the binding of ammonia with some uncertain source of carbon, occurs in the liver where hypoxanthine is formed.

2. The final conversion into uric acid, which takes place in the kidney (or pancreas), is an oxidation catalysed by xanthine oxidase.

3. There is evidence suggesting that hypoxanthine is also an intermediate in other birds.

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CXCVII. AN INVESTIGATION OF THE RATES OF DIGESTION OF STARCHES AND GLYCOGEN AND THE BEARING ON THE CHEMICAL CONSTITUTION.

I. ACTION OF AMYLASES ON STARCHES AND GLYCOGEN.

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Starches from different sources show very different rates of hydrolysis by amylases. This is presumably due to differences in the composition of the starch granules. Dietetically, however, starches are usually considered of equivalent food value, provided that they all undergo complete digestion. Having found that potato and rice starches are hydrolysed at very different rates, as judged by the achromic point, and being unable to find in dietetical and chemical literature information concerning this, we decided to investigate the rates of digestion and of the formation of the intermediate hydrolysis products of the starches which are incorporated in our diet. Glycogen was later included in the investigation. In this way it was hoped to gain information concerning the differences in chemical constitution of the different starches and their relative digestibilities.

Concerning the chemical structure of starch little need be said here. The work of Samec [1927], Ling & Nanji [1923], Schardinger [1909; 1911], Pringsheim & Wolfsohn [1924] all supports Maquenne & Roux's [1906] theory that the starch granule consists of two constituents (termed by these workers amylopectin and amylose). The work of Karrer & Nägeli [1921, 1, 2] and of Haworth [1931; 1932; 1935], Hirst *et al.* [1932], on the other hand, supports the now more generally adopted view that starch is homogeneous in structure. Amylose and amylopectin are reported to be formed by successive depolymerization and repolymerization of natural starch and their distinctive characteristics to arise from differences in physical character or from admixture with other substances.

Most workers consider that the unrestricted action of maltase-free amylases on starch leads to the formation of one sugar only, namely maltose, and that by the prolonged action of amylase there is probably at least 80% conversion into α-glucosidic maltose. Certain early workers, however, namely Lintner & Düll [1892; 1893] considered that *iso*maltose is formed in addition to maltose. *iso*-Maltose, as prepared by these workers by the action of malt diastase on potato starch, has a reducing power of 80% of that of maltose, $[\alpha]_D$ 140°, and forms an osazone of M.P. 151° which crystallizes in spherical groups of very fine light yellow needles and is very much less soluble in water and alcohol than maltosazone. Syniewski [1902] confirmed the above work and claimed to have isolated pure *iso*maltose (termed by this worker "dextrinose"). Brown & Morris [1895] showed that Lintner's *iso*maltosazone is not a separate chemical entity but is only maltosazone altered in crystalline form and M.P. by admixture with an amorphous osazone of a simple dextrin. Ling & Baker [1895] are also of this opinion. Pottevin [1899] interpreted the amylolytic hydrolysis of starch as due to partial hydrolysis of the starch granule by means of an enzyme "amylase", into a series of dextrins, ranging from amylodextrins, giving a blue colour with iodine and exhibiting slight Fehling's reducing properties, to achroodextrins or simple dextrins, giving no colour with iodine and having high Fehling's reducing properties. A second enzyme "dextrinase" then converts these dextrins into maltose. According to Chrząszcz [1911], an amylase contains separate depolymerizing, dextrinizing and saccharifying enzymes, which view was later supported by Pringsheim [1924], but this assumption does not seem to be essential. Nishimura [1928] regards amylases as being composed of one enzyme only, which saccharifies and depolymerizes, the latter notably in the presence of a co-enzyme. Olsson [1923] showed that the saccharifying enzyme is different from the liquefying enzyme because the addition of certain poisons reduced the liquefying action without affecting the saccharifying power. Kendall & Sherman [1910] found that the liquefying action is predominant with pancreatic amylase and the saccharifying action with malt amylase. These two enzyme components have now been successfully separated by Van Klinkenberg [1934]. Karrer [1921] considered diastatic action to consist of two phases, depolymerization and hydration, the latter phase involving the hydrolysis of maltose anhydride to maltose. The saccharification of depolymerized amylose and amylopectin does not proceed at the same rate. Amylose smoothly and quickly gives rise to the theoretical amount of maltose, whereas the saccharification of amylopectin is slower and usually ceases when about 78 % of the theoretical yield of maltose is produced. The remaining residual dextrin can, according to Pringsheim & Beiser [1924] be converted into maltose if a co-enzyme (present in yeast) is added. Kuhn [1924] divided amylases into two groups, the α- and β-amylases according to the point of attack of the starch molecule. α-Amylases produce α-glucosidic maltose as the end-product of starch degradation and β-amylases produce β-glucosidic maltose initially. Amylases from animal sources and takadiastase are α-amylases and vegetable amylases, with the exception of takadiastase, are β-amylases. Kuhn concludes that in view of the behaviour of the starch granule towards plant and animal amylases, the starch molecule consists of alternating α- and β-glucosidic linkages. If both α- and β-glucosidic groupings occur in the starch molecule it should be possible to obtain glucose from starch if both α- and β-amylases are made to act on starch paste. Pringsheim & Liebowitz [1925] showed this to be true. Ohlsson [1930] showed that his "dextrinogenamylase" from malt was identical with Kuhn's α-amylase and his "saccharogenamylase" with Kuhn's β-amylase. As judged from the relative permanence of the starch-iodine reaction, Ohlsson concluded that the former enzyme breaks down starch into progressively smaller molecules until maltose is produced, whereas the latter enzyme detaches maltose in successive stages. Van Klinkenberg [1932], in opposition to Kuhn, regards the α- and β-glucosidic linkages as occurring not in the same molecule but in different molecules, in α- and β-starch, respectively.

Little work has been done on the rates of digestion of different starches and the results that are quoted are very discrepant. The chief reason for this discrepancy is that two different methods are used for determining the amount of digestion that has occurred. These two methods, the achromic point and a sugar determination, are actually estimating different things. The achromic point determines when all the starch has been broken down at least as far as achroodextrin, although this does not necessarily mean that there is any sugar present. A sugar determination, on the other hand, estimates the maltose present and also any reducing dextrins, but there may at the same time be a considerable amount of unchanged polysaccharide left. In order to get an accurate conception of the amount of starch degradation that has taken place at any time it is necessary to determine at least four factors; the achromic point, the unchanged polysaccharide, the total reducing power and the reducing power due to maltose alone. O'Sullivan [1904] found that a much lower percentage of maltose was formed by the action of malt amylase on potato starch than on cereal starches. Stone [1904] found that potato starch was more readily digested by pancreatin than were cereal starches. Ford [1904] showed that when rice, barley, maize, wheat and potato starches were purified by the same method, they were digested equally by malt extracts. Nagao [1911] found that wheat and rye starches were hydrolysed at equal rates by pancreatic amylase and that oat and barley starches were digested at approximately the same rate, but that the rate of hydrolysis of the latter two was considerably higher than that of wheat and rye. Sherman *et al.* [1919] showed that, when similarly purified by washing with dilute alkali to extract any gluten, wheat, maize and rice starches were hydrolysed at equal rates. Potato starch generally showed a rate of hydrolysis equal to or slightly greater than the cereal starches. These experiments were performed with both pancreatic and salivary amylases. The percentage hydrolysis was determined in each case at the end of 30 min. by estimating the reducing sugar by Fehling's method.

Concerning the breakdown of glycogen, Musculus & Mering [1879] showed that glycogen gave the same hydrolysis products as starch. Cremer [1894] found that the amylolytic hydrolysis of glycogen was much slower than starch. Tebb [1897] found that when glycogen was digested by ptyalin, amylopsin and malt diastase, both maltose and *iso*maltose were formed. Osborne & Zobel [1903] found that both pancreatic and malt amylases formed *iso*maltose alone as the end-product, whereas both takadiastase and salivary amylase produced glucose in addition to *iso*maltose. Horton [1913] showed that the hydrolysis of glycogen by pancreatic amylase proceeded much faster in the early stages, but the subsequent breakdown of dextrin was very slow and there was seldom a quantitative conversion into maltose. Pringsheim's view that amylopectin and glycogen are identical was further substantiated by the work of Pringsheim & Beiser [1924] who showed that a co-enzyme was necessary for the complete hydrolysis of glycogen, just as in the case of amylopectin. Barbour [1929] showed that with salivary amylase the chief end-product was *iso*maltose, and there was also occasionally glucose present, but never any maltose. With pancreatic amylase, both *iso*maltose and glucose were formed. He found that there was a serial degradation of glycogen with pancreatic amylase, the increased reducing power corresponding with disappearance of glycogen, whereas with salivary amylase there was no such correspondence.

In view of these rather discordant results and conclusions the present investigation was carried out using potato, maize, wheat and rice starches (all of these having been obtained from B.D.H.) and glycogen (Kahlbaum).

The ash and P contents of all the samples were determined; as a further test of purity nitrogen analyses were performed but the results were negative in all cases. The values obtained are given in the following table, all the estimations being performed on the dried material.

Polysaccharide	% Ash	% P
Maize starch	0·084	0·055
Wheat starch	0·185	0·142
Potato starch	0·285	0·119
Rice starch	0·284	0·068
Glycogen	1·100	0·298

The ash in each case gave a positive iron reaction. The P was determined by Neumann's method using 0·5–2·0 g. of dried material. The results are of the same order as those obtained by Samec & Haerdtl [1920] and the higher value obtained for glycogen substantiates the generally adopted view that glycogen has a higher electrolyte content than starches.

Achromic point.

The achromic points were determined for each polysaccharide, using a 1% solution and hydrolysing it in turn with saliva, takadiastase, malt diastase and a solution of "Holadin" (a pancreatic preparation particularly rich in amylopsin) at the appropriate optimum *p*H. Whichever amylase was used, the achromic points were always in the same order, this point being reached very much more quickly with potato starch and glycogen than with rice, wheat and maize starches, these latter three, however, all giving the achromic point at approximately the same time.

	Starch				
Amylase	Potato sec.	Maize hours	Rice hours	Wheat hours	Glycogen
Salivary	20	1·5	1·5	1·5	Few sec.
Pancreatic	5	2·25	2·25	2·25	Immediate
Takadiastase	20	4	4	4	Few sec.
Malt	240	3	3	3	2 min.

As will be seen from the following results, the achromic point is a very unreliable index of the amount of digestion that has taken place.

According to Van Klinkenberg [1934] iodine-colouring atomic groups are present in α-starch only and the iodine reaction therefore only disappears by the action of α-amylase.

Rate of degradation of the different starches and of glycogen.

The course of the enzymic hydrolysis of glycogen and of each of the starches was followed using pancreatic amylase ("Holadin"). The salivary and malt digestions of wheat starch and glycogen were also followed.

The maltase activity of all the enzyme preparations was tested by incubating 2% maltose solutions with relatively large amounts of the enzyme and testing for glucose by an osazone test after 3 days. Except with the malt preparation, glucose was produced in every case. On diminishing the concentration of enzyme, however, no glucose was formed even with concentrations of enzyme ten times as great as those used in the subsequent hydrolyses. It is concluded that these enzyme preparations contain only traces of maltase which are ineffective in the low concentrations in which they were employed and would therefore be overlooked in most cases.

The following estimations were performed:

(1) estimation of unchanged polysaccharide;

(2) estimation of total reducing power using the Wood-Ost method;

(3) estimation of reducing power due to sugar alone using the same method.

In the later experiments Hanes's [1929] method of sugar estimation was substituted for that of Wood-Ost. From these data it is possible to calculate the amount of polysaccharide that has been broken down, the amounts of reducing dextrin and maltose formed, and, by deduction, the amount of non-reducing dextrin.

These three estimations were performed on samples of the digest immediately after mixing and 15, 30, 60 or 90, 120, 180, 330 min., 24 or 72 hours after the beginning of the experiment.

An osazone test was also performed on samples from each digest, after alcohol precipitation of any residual starch and dextrins followed by evaporation of the alcohol from the filtrate. In no case was a typical maltosazone observed. Most frequently the osazone consisted of relatively large rosettes of very fine needles but very often these needles were tufted at their free extremities. It seems impossible to place any reliance on the appearance of the osazone; either the crystalline form typical of maltosazone had become modified by admixture with another substance (perhaps dextrins which had not been completely removed by alcohol precipitation) or else maltose is not the sole product of hydrolysis.

Different methods were employed for the estimation of starch and glycogen.

The starch was estimated by a modification of the von Fellenberg method [1916]. This method depends on the principle that starch dissolves in concentrated $CaCl_2$ solution, from which it can be precipitated by iodine. Protein, which might be present in small amounts, is insoluble in $CaCl_2$ solutions and by dextrins are not precipitated from $CaCl_2$ solutions by iodine.

The digestion mixture in the case of the pancreatic digestion consisted of 225 ml. of 1 % starch solution in 0·5 % NaCl, 70 ml. of borate[1] buffer of pH 6·8 and 5 ml. of 0·1 % solution of holadin in distilled water. This mixture was left in a thermostat at 37° and samples were removed at the time intervals stated above.

The total reducing power was determined by the Wood-Ost method, introducing 5 ml. of the digest into 20 ml. of the Wood-Ost solution. This was performed in duplicate.

In order to determine the reducing power due to maltose alone, 10 ml. of the digest were removed, boiled to inactivate the enzyme, cooled and made up to 50 ml. in a graduated flask with absolute alcohol. This was left overnight, filtered and 20 ml. portions of the filtrate used for the Wood-Ost determination. In many cases a further estimation was carried out. A portion of the alcohol filtrate, which should contain maltose alone, was heated on a water-bath to drive off all the alcohol and the reducing power determined after acid hydrolysis. This should indicate whether maltose is the sole sugar formed.

Furthermore, in a few cases, polarimetric determinations were carried out on the alcohol filtrates, after the alcohol had been driven off and the subsequent solution considerably concentrated and the turbidity removed, if necessary, by the addition of a small amount of basic lead acetate.

At the end of each experiment the digest was tested for lactic acid by Uffelmann's reagent. This test was in all cases negative.

The digestion mixture for the glycogen hydrolysis was the same as for the starches. The total reducing power and the reducing power due to sugar were determined in exactly the same way as described for starch. In the determination of the residual glycogen, the observations of Good *et al.* [1933], that the optimum concentration of alcohol for the precipitation of glycogen is 50 % and of Osterberg [1930] that this precipitation is assisted by the use of Na_2SO_4, which carries down mechanically the precipitated glycogen with the precipitate of Na_2SO_4, were made use of. For the glycogen determinations 3 ml. samples of the digest were employed and the glucose, after acid hydrolysis was estimated by Hanes's method.

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[1] Phosphate buffers cannot be substituted for borate buffers, since it was found that boiling the starch and phosphate mixture with $CaCl_2$ led to the complete disappearance of the starch.

The results are represented diagrammatically (Fig. 1) and in addition three graphs are given plotting respectively the percentage disappearance of polysaccharide, the percentage formation of reducing dextrin and maltose, and the

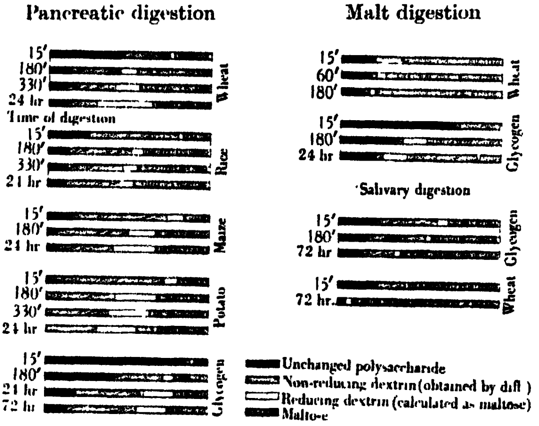

FIG. 1. Representing the formation of the products of digestion of starches and glycogen.

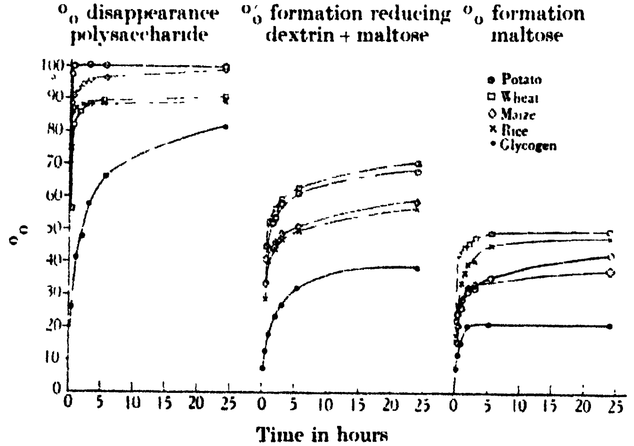

FIG. 2. Digestion of starches and glycogen by pancreatic amylase.

percentage formation of maltose against time. (The time axis is shortened for economy of space.)

Nature of sugar formed on hydrolysis.

As has already been stated most observers agree that maltose is the sole sugar formed on hydrolysis of starch or glycogen by amylases. The present results are not entirely in agreement with this. The same sugar was formed from all the starches and from glycogen but as judged from the appearance of the osazone, the amount of glucose formed on acid hydrolysis and the optical rotation of the sugar, this sugar could not be identified with maltose. The appearance of the osazone has already been described. On acid hydrolysis of the digest, after precipitation of the unchanged polysaccharide and dextrins with alcohol, 120–140 % yield of glucose was obtained (assuming that the sugar before hydrolysis was maltose). This means that the reducing power of the sugar before acid hydrolysis was only 70–85 % that of maltose. In no case, as judged by the osazone was any glucose present, but if any were present this would mean that the other sugar would have a reducing power even further removed from that of maltose than the value given above.

In a few cases only was the optical rotation determined, but when this was done it was invariably found that there was lack of agreement between the optical rotation and the reducing power (assuming the sugar to be maltose only). The optical rotation was in each case 124·5 % that of maltose. This value is very similar to that given for α-maltose, but this possibility is excluded because no mutarotation was observed after treatment with alkali. The constancy of this figure obtained both from potato and wheat starches at different stages of hydrolysis indicates that only one sugar could be present. If a mixture of sugars were present, it is extremely unlikely that they would be present in exactly the same proportions under different conditions. These results might be explained by the presence of reducing dextrins, but since the final concentration of alcohol before filtration of the unchanged polysaccharide was 80 % this seems improbable.

The appearance of the osazone and the reducing power corresponded fairly closely with those of *iso*maltose as described by Lintner & Düll. The specific rotatory power, however, was different. The figure given for *iso*maltose is $[\alpha]_D = 140°$, which is only very slightly different from that of maltose.

DISCUSSION OF RESULTS.

There are at least four different criteria of the rate of degradation of the polysaccharides:

(1) rate of disappearance of polysaccharide:
(2) rate of formation of maltose and reducing dextrin;
(3) rate of formation of maltose;
(4) achromic point.

The following table represents the order of these events, the polysaccharides being arranged in order of decreased speed of hydrolysis by pancreatic amylase as indicated by the different criteria.

Achromic point	Rate of disappearance of polysaccharide	Rate of formation of reducing dextrin and maltose	Rate of formation of maltose
Glycogen	Potato, maize	Wheat, potato	Wheat, rice
Potato	Wheat, rice	Maize, rice	Potato
Maize, wheat, rice	Glycogen	Glycogen	Maize
			Glycogen

It is difficult to reconcile the very quick achromic point of glycogen with its slow rate of breakdown and formation of dextrin and maltose. It must be the figures for the achromic point which are at fault, since it was found very difficult to determine this value when very weak glycogen solutions were used, the colour being only with difficulty distinguishable from that of a weak iodine solution.

It is interesting to note that in the case of glycogen, the rate of formation of maltose reached a steady state after 2 hours, although the formation of reducing dextrin and the disappearance of glycogen were still occurring. (The decrease in the 24 hours value for maltose seen in the results for wheat and maize starches is presumably due to fermentation.)

In the case of the salivary digestion of glycogen and wheat starch, although the salivary amylase was obviously relatively very much stronger than the pancreatic amylase, the results are in the same order, the figures for glycogen being in every case smaller than those for wheat starch.

In the case of the digestion of glycogen and wheat starch with malt there is a slightly greater breakdown of glycogen than with pancreatic and salivary amylases. Malt contains chiefly β-amylase, but this does not warrant the assumption that glycogen contains a preponderance of β-linkages. The breakdown of glycogen by pancreatic, salivary and malt amylases is essentially similar in character.

These differences in the hydrolysis rates of the different starches must be due to differences in the chemical constitution of the starch granules, since they cannot be attributed to differences in the enzyme systems.

The rate of hydrolysis of all the starches is very much quicker than that of glycogen.

Factors which could influence the rate of breakdown.

(1) *Molecular dimensions of the polysaccharide.* The smaller the molecular size, the quicker one would expect the breakdown to be. But the size of the glycogen molecule is (according to Haworth) approximately half that of the starch molecule.

(2) *Proportion of amylopectin to amylose.* According to most modern workers the starch granule is homogeneous. But supposing that two constituents are present, the advocates of this theory agree with the original view of Maquenne & Roux [1906] that the viscosity of a starch solution is produced by the amylopectin. According to this view, potato starch, which gives the most viscous solution, should show the lowest rate of hydrolysis, since amylopectin is considered to be hydrolysed more slowly than amylose. This, however, is not the case, potato starch showing, if anything, a higher rate of hydrolysis in the initial stages than the other starches. The lower rate of hydrolysis of glycogen, however, could be accounted for if, as claimed by Pringsheim, glycogen consists of amylopectin.

(3) *Inorganic content of polysaccharide.* According to Samec & Haerdtl [1920] a low rate of hydrolysis can be correlated with a high P content. Potato and wheat starches have much higher P contents than the other starches, but their rates of hydrolysis are no lower. The lower rate of hydrolysis of glycogen, however, could be explained according to this theory by its high P content.

(4) *Method of breakdown.* As has already been mentioned Ohlsson considers that α-amylases break down starch into smaller and smaller molecules until maltose is finally produced whereas β-amylases detach maltose successively. In the salivary and pancreatic digestions of glycogen and the various starches employed in this investigation, an early appearance of maltose was invariably observed. When malt, which contains β-amylase in addition to α-amylase, was used as the hydrolysing agent, no increased formation of maltose was observed. These results therefore do not substantiate the findings of Ohlsson. Moreover, Ohlsson's work was based on the relative permanence of the starch-iodine reaction, and since it is not known with certainty that this colour is a function of the molecular dimensions, his conclusions seem rather unreliable.

In order to ascertain which of the above-mentioned methods of breakdown was involved or which method was predominant, the following ratio was calculated for each digestion mixture throughout each hydrolysis:

$$\frac{\text{Polysaccharide destroyed in mg./100 ml.}}{\text{Maltose formed in mg./100 ml.}}.$$

If this ratio remains constant throughout a hydrolysis it would indicate that maltose was liberated successively. If, on the other hand, the ratio decreases as digestion proceeds it would mean that maltose is formed only, or principally, in the final breakdown of the simple dextrins. These ratios, calculated from determinations made at 15 min. from the beginning of each experiment and at the subsequent intervals already mentioned, are tabulated below. The values cited represent values for this ratio obtained at the beginning, middle and end, respectively, of each experiment:

Substrate	Pancreatic amylase			Malt diastase			Salivary amylase		
Glycogen	2·16	2·46	3·15	1·24	1·40	1·50	2·52	1·98	1·39
Potato	4·00	3·52	2·78	—	—	—	—	—	—
Wheat	1·87	1·83	1·86	1·27	1·13	1·09	1·39	—	1·04
Rice	2·60	2·56	1·89	—	—	—	—	—	—
Maize	4·38	2·89	2·70	—	—	—	—	—	—

The great variability of this ratio probably indicates that both methods of breakdown are occurring simultaneously. It is difficult to accept the deduction that the hydrolysis of wheat starch by pancreatic amylase is the one instance amongst all those cited in which the former method of breakdown alone occurs. An increase in this ratio as hydrolysis proceeds, observed with glycogen in two out of the three cases, seems very difficult to explain, especially as there was no glycogen resynthesis.

In the case of glycogen, the formation of maltose from dextrin appears to be very slow, which indicates that the hydrolysis is retarded at this stage, suggesting a reduced saccharification process. But since the same enzymes were used in the starch and glycogen digestions, this cannot be accounted for by the enzyme. Perhaps, as suggested by Pringsheim, a co-enzyme is necessary for the complete hydrolysis of glycogen.

(5) *Nature of linkages in polysaccharide.* As discussed in the previous section, the slow rate of breakdown of glycogen cannot be explained by assuming a preponderance of β-linkages in the glycogen molecule.

According to Van Klinkenberg the iodine reaction is characteristic of α-starch and only disappears by the action of α-amylases. The very rapid attainment of the achromic point observed with glycogen appears at first sight to substantiate Van Klinkenberg's view that glycogen is α-starch. But this does not explain the slow breakdown of glycogen in comparison with the starches. If glycogen consisted entirely of α-starch, a very quick breakdown by α-amylases would be expected.

It is thus seen that the theories of starch and glycogen structure so far enunciated do not explain satisfactorily the results of the amylolytic hydrolysis of these polysaccharides. There must be some difference in structure between the starch and glycogen molecules to account for the much lower rate of hydrolysis of the smaller glycogen molecule. There are also probably slight differences in structure between the different starches. It is also difficult to accept the identity of the starch and glycogen molecules as advocated by Haworth.

SUMMARY.

1. The course of digestion of potato, wheat, maize and rice starches and of glycogen by pancreatic amylase and in addition of wheat starch and glycogen by salivary amylase and malt diastase have been followed quantitatively.

2. The estimations included determinations of the achromic point, the residual polysaccharide, the total reducing power and the reducing power after the precipitation of the dextrins. Polarimetric observations were also made in a few cases.

3. The relative rates of formation of the various hydrolysis products were different with the different starches. The rate of hydrolysis of glycogen was in every case very much lower than that of the starches.

4. The same end-product was formed from all the starches and from glycogen.

5. The properties of the reducing sugar formed as end-product did not correspond with those of maltose, or, in all respects, with those of *iso*maltose.

6. Glucose was not formed in any of the above digestions, or in the case of pancreatic amylase over the range *p*H 5·0–7·5. When, however, a very large amount of enzyme was employed, some glucose was produced.

7. The bearing of these results on the chemical constitution of the starch and glycogen molecules and on the method of breakdown is discussed.

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CXCVIII. BIOCHEMICAL STUDIES IN THE NITROGEN METABOLISM OF THE APPLE FRUIT.

III. PRELIMINARY EXPERIMENTS ON THE EFFECT OF INJECTING NITROGENOUS COMPOUNDS INTO APPLE TREES ON THE COMPOSITION OF THE FRUIT. (WITH AN APPENDIX DESCRIBING A NEW VACUUM EXTRACTOR.)

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The nitrogen content of the mature[1] apple varies markedly both in total amount and in the manner in which it is distributed amongst the compounds in which it occurs [Hulme, 1932; 1934: and unpublished data]. The possible connexion between these initial differences of composition and the behaviour of the fractions of the nitrogen and of the fruit in general, when detached from the tree and kept under various conditions of environment, is being studied. The purpose of the present investigation was to determine whether the injection of nitrogenous compounds into the tree is followed by a modification of the nitrogenous composition of the fruit. In this event injection would be a valuable method for modifying at will the nitrogenous composition of the fruit as a preliminary to the above study. Methods have been developed for injecting whole trees so that each branch received its proper share; further, separate branches, twigs, leaves or portions of leaves may each be injected with a different solution without risk of the injected substance reaching any part of the tree other than the one for which it is intended.

Preliminary papers have already appeared [Roach, 1933; 1934, 1, 2, 3: 1935; Hearman *et al.* 1935] and the details of the methods are at present being prepared for publication. Such methods would make it possible to treat fruit at any stage in its development without affecting the relations between the tree and the soil and without undue interference with the physiology of the tree.

Experimental.

(1) *Material and details of tree-injection.*

Six bush trees of variety King Edward VII budded on Malling Type IX rootstock and four trees of variety Bascombe Mystery budded on Malling Type V rootstock were used for the experiment. The trees were planted as maidens in

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[1] "Maturity" is here used in the somewhat loose sense of the stage at which the fruit is normally gathered for commercial purposes. It is not yet clear to what definite stage (if any) in the physiology of the fruit this approximates.

December 1921. The King Edward VII trees varied in branch spread from 2·1 to 2·8 m. and the Bascombe Mystery ones from 3·2 to 3·7 m. These trees varied in size and in other respects but they were amongst the few trees which escaped the disastrous frost of the night of 16–17 May 1935, and were used in the absence of any better material; they proved sufficiently uniform for the present purpose.

Injection. The injection of each tree was carried out as follows: A hole of ¼ in. diameter was bored through the main stem just below the crotch and so placed that each end came below the junction between two branches and so that the parts of the top on either side of the hole were as nearly equal as possible. One end of this hole was connected with rubber tubing to the reservoir containing the liquid which was supported a few inches above the level of the hole, and into the other end of the hole a short length of rubber tubing was inserted (see Fig. 1). Both pieces of tubing were inserted only to the depth of the bark. The joints between the tubing and the bark were made watertight by painting with a molten solution of rubber and paraffin-wax. The siphon was started by sucking the short rubber tube, which was plugged when the liquid came through. The liquid was absorbed in from 1 to 3 days. Two Bascombe Mystery trees were injected a second time through holes at right angles to the first ones (see Table II).

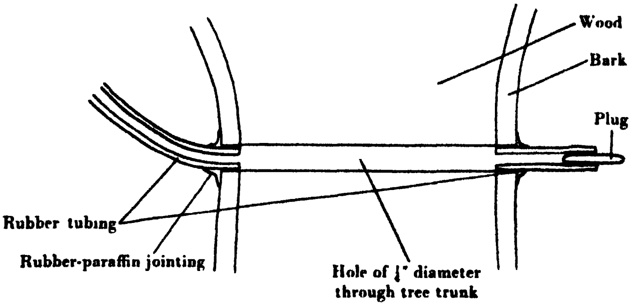

Fig. 1.

Substances injected. The substances injected will be seen from Tables I and II. Each solution contained a standard amount of nitrogen per unit volume.

Dosage. A system for deciding the correct dosage for trees according to their size has not yet been worked out; the volumes of solution given were decided on by inspecting the trees and comparing them with others injected in the past, the aim being to give each tree an amount of nitrogen proportional to its size.

Leaf damage. Traces of damage appeared on the leaves of most of the trees the day after the injection was finished but this was of no importance; the foliage of the Bascombe Mystery tree injected with ammonium nitrate was, however, heavily damaged. This damage has been described elsewhere [Hearman *et al.* 1935]. Some leaves fell but later fresh leaves expanded and the tree again had a normal amount of foliage. Reference has already been made to the high toxicity of nitrate-nitrogen as compared with urea-nitrogen [Thomas & Roach, 1934].

(2) *Sampling of the material and its preparation for analysis.*

At the time of gathering the fruits were greatly reduced in number owing to falls, chiefly as a result of codling moth attack. All the fruits were gathered from each tree, any seriously blemished fruit being discarded. It was not necessary to discard any complete apples in the case of the King Edward VII variety, but one or two fruits per tree had to be eliminated in the case of the Bascombe Mystery trees. Where only a small amount of the apple was damaged the damaged portion was cut out immediately before freezing and allowance made in calculating the weight of pulp and peel.

For the King Edward VII trees, details of the fruit as gathered are given in Table I which also includes details of the solutions injected into the tree.

Table I.

Sample "(K.E.)"	Tree	Injection 15. vii. 35	No. of fruits	Av. wt. of apple g.	Total wt. of pulp* g.	Total wt. of peel g.	Ratio pulp/peel
C (1)	6 + 12	None } Controls	32	124·4	3368	420	8·02
C (2)	12	None	32	130·0	3508	457	7·68
N	2	10 l. 0·33 % NH_4NO_3	30	124·3	3163	409	7·73
A	4	7 l. 0·55 % asparagine	30	126·6	3247	410	7·92
U (1)	10	10 l. 0·25 % urea	30	134·0	3400	430	7·91
U (2)	10	10 l. 0·25 % urea	2	155·7	1599	201	7·91
	8	10 l. 0·25 % urea	10				

* Corrected for seeds and bad areas removed. The amount of the latter was between 5 and 8 % of the whole of each sample. It was fairly uniformly distributed between the fruits of all the trees. The amount of peel tissue concerned was negligible.

In the case of the Bascombe Mystery trees (see Table II) the number of fruits per tree was larger although considerably less than at the time of injection. The larger number of fruits enabled more than one sample to be made up from each tree (except tree 3, urea treatment) and an additional sample was placed in the dark for 54 days at 9·5°, so that the change in the nitrogenous fractions after detachment from the tree could be studied. The concentrations of the various solutions injected were the same as for the King Edward trees (Table I).

Table II.

		Injection						
Sample	Tree	15. vii. 35	1. x. 35	No. of fruits	Av. wt. of apple g.	Total wt. pulp g.	Total wt. peel g.	Ratio Pulp/Peel
CO (1)	1	None (control)		26	62·6	1335	207	6·45
CO (2)	1			30	69·0	1730	268	6·46
CI*	1			30	69·1	1607	270	5·95
NO	2	15 l. NH_4NO_3		30	52·9	1298	223	5·82
NI*	2			30	52·6	1205	226	5·58
UO	3	15 l. urea	10 l. urea	34	73·6	2064	330	6·26
AO	4	13 l. asparagine	11 l. asparagine	29	61·8	1470	236	6·23
AI*	4			28	62·7	1340	234	5·73

* Samples placed at 9·5° for 54 days. During this period there was, of course, some loss of weight as moisture and CO_2. This loss of weight was measured and allowed for in the calculation of results.

From the values of the ratios of pulp/peel given in Table II it would appear, as might be expected, that the pulp tissue undergoes a greater loss in weight during storage than does the peel.

The material was frozen to −20°, peeled and prepared for analysis by the method previously described [Hulme, 1936].

(3) *Methods of analysis.*

The various fractions of the nitrogen and the titratable acidity were estimated by the methods already described [Hulme, 1936], with the exception that in the extraction of the soluble nitrogen an improved form of continuous vacuum extractor was used. This extractor, when used in conjunction with Naumann evaporators [Naumann, 1935] to remove the excess alcohol from the extracts, greatly reduced the time required to obtain the final extracts. A brief description of this new extractor is given in an appendix (p. 1402).

Urea was estimated in several samples—chiefly those from trees injected with urea—by a micro-method based on the urease method of Yee & Davis [1935].

It is, perhaps, improbable that urea injected into the tree would reach the fruits as such, unless it was in excess of that with which the leaves could deal. Nevertheless, it is important to make certain of this point, since, apart from the direct interest of the presence of urea in the fruits, appreciable amounts of urea in the extracts would seriously interfere with the estimations of amide and amino-nitrogen [Chibnall & Westall, 1932]. In no case were amounts of urea found greater than 0·3 % of the total nitrogen, and the fruit from trees injected with urea gave values similar to those injected with asparagine.

Glutamine determinations were made on the extract from sample K.E. C (1) by the method of Vickery *et al.* [1935] but this amide was found to be absent.

RESULTS.

The results obtained for the apples as gathered from the trees are given, for both varieties, in Table III, A, B, and for the stored fruits of the Bascombe Mystery variety in Table III C.

It is obvious from Table III that the injections have had a considerable effect on the total nitrogen content of the fruits. In all cases the total nitrogen of the fruits from injected trees is greater than in those from the uninjected ones. On the whole the figures for the pulp and the peel show the same general trend. The most striking fact is that, whilst the total nitrogen has increased considerably, the increase is mainly accounted for by an increase in the non-protein fraction. Whilst in the case of the King Edward variety the absolute amount of protein (even allowing for the difference in size of the fruits) is slightly higher in all the injected trees, the relative amount of protein (i.e. protein as % of the total nitrogen) is considerably decreased. The results for sample U (2) are probably "abnormal" since the sample was mainly composed of fruits from tree 8 which bore, at the time of gathering, only 10 fruits and these were much larger in size than those from any of the other trees of this variety.

The increase in soluble nitrogen is chiefly in the form of asparagine; the fraction of the soluble nitrogen in the form of amino-acids shows relatively little variation throughout the series. The same is even more strikingly true for the Bascombe Mystery series. This is in agreement with the view of Prianischnikow and many modern authors that asparagine may function as a means of storing excess nitrogen in a form innocuous to the plant.

In both series injection of urea appears to result in a small increase in the proportion of amino-acids and a lowering of the relative amount of protein. The two effects are not, however, equal quantitatively. The Bascombe Mystery fruits injected with ammonium nitrate are, as stated above, probably abnormal, and the very low proportion of protein which they contain is possibly due to the temporary damage to the tree following injection.

Table III.

	Sample	Total N		Protein-N		Total soluble N		Free NH_3-N	Asparagine-N		Amino-acid-N		"Rest" N		Titratable
		mg./ apple	% fresh weight	mg./ apple	% total N	mg./ apple	% total N	% total N	mg./ apple	% total N	mg./ apple	% total N	% total N	*p*H of extracts	acid (as malic) mg./apple
A. King Edward VII apples as gathered.															
Pulp	K.E. C (1)	45·8	0·0435	24·5	53·4	21·3	46·6	0·19	10·3	22·5	5·8	12·7	11·1	2·79	1297
Peel		12·4	0·0943	10·5	84·8	1·9	15·2	0·19	0·7	5·4	0·6	4·9	4·7	—	147
Pulp	K.E. C (2)	46·8	0·0428	25·0	53·4	21·8	46·6	0·25	11·7	24·9	6·2	13·2	8·2	2·83	1233
Peel		12·9	0·0904	10·9	84·4	2·0	15·6	0·20	0·8	6·0	0·7	5·1	4·3	—	149
Pulp	K.E. N	56·3	0·0534	27·8	49·4	28·5	50·7	0·26	16·4	29·1	8·9	15·8	5·5	2·78	1340
Peel		13·4	0·0983	11·0	82·5	2·3	17·5	0·35	0·9	6·8	0·8	5·7	4·7	—	165
Pulp	K.E. U (1)	64·6	0·0570	28·7	44·4	36·0	55·7	0·18	23·2	35·9	10·0	15·5	4·1	—	1348
Peel		14·6	0·1013	11·7	80·3	2·9	19·7	0·22	1·2	8·2	1·1	7·4	3·9	—	170
Pulp	K.E. U (2)	89·4	0·0672	28·7	32·1	60·7	67·9	0·17	37·4	41·8	13·6	15·2	10·7	2·79	1804
Pulp	K.E. A	53·8	0·0497	25·8	48·1	28·0	51·9	0·24	16·1	29·9	7·4	13·8	8·0	2·76	1340
Peel		13·2	0·0963	10·8	82·2	2·4	17·9	0·25	0·9	6·8	1·0	7·2	3·5	2·98	161
B. Bascombe Mystery apples as gathered.															
Pulp	B.M. C (1)	20·4	0·0397	11·4	55·7	9·0	44·3	0·34	4·7	23·1	2·4	11·8	9·1	2·95	402
Peel		7·6	0·0955	6·6	87·2	1·0	12·8	0·33	0·3	4·5	0·3	4·3	3·7	—	54
Pulp	B.M. C (2)	22·6	0·0393	12·5	55·3	10·1	44·7	0·35	5·3	23·4	2·7	11·9	9·0	2·96	414
Peel		8·4	0·0943	7·3	87·0	1·1	13·0	0·21	0·4	4·4	0·4	5·0	3·4	—	59
Pulp	B.M. N	30·9	0·0713	10·4	33·7	20·5	66·3	0·16	14·4	46·6	3·3	10·8	8·7	2·90	310
Peel		8·4	0·1125	6·1	73·5	2·2	26·5	0·32	1·3	15·7	0·4	4·8	5·7	—	51
Pulp	B.M. U	32·0	0·0527	15·1	47·2	16·9	52·8	0·29	10·1	31·5	4·1	12·7	8·3	—	529
Peel		9·5	0·0978	7·7	80·7	1·8	19·4	0·35	0·8	8·3	0·6	6·0	4·7	—	78
Pulp	B.M. A	29·2	0·0576	13·4	45·8	15·8	54·2	0·14	9·7	33·2	3·2	10·8	10·1	2·89	487
Peel		7·9	0·0970	6·3	79·7	1·6	20·3	0·27	0·7	8·8	0·4	5·0	6·2	—	68
C. Bascombe Mystery apples after storage.															
Pulp	B.M. C (1)	21·6	0·0378	14·4	66·5	7·2	33·5	0·83	4·1	19·0	1·8	8·5	5·2	2·96	357
Peel		9·1	0·0949	8·0	87·1	1·2	12·9	0·46	0·4	4·3	0·2	2·2	5·9	—	48
Pulp	B.M. N (1)	28·7	0·0669	9·6	33·6	19·1	66·4	0·80	14·0	48·8	3·0	10·4	6·4	3·11	220
Peel		9·6	0·1246	7·2	75·0	2·4	25·0	0·49	1·6	16·4	0·3	3·4	4·7	—	30
Pulp	B.M. A (1)	30·3	0·0596	12·9	42·5	17·4	57·5	0·89	11·5	37·9	2·7	9·0	9·7	3·01	360
Peel		9·9	0·1110	7·9	79·6	2·0	20·4	0·50	1·1	11·1	0·3	3·1	5·7	—	45

We may next turn to a brief consideration of the observed changes in the nitrogen fractions of the Bascombe Mystery fruits during their short period of storage (Table III c). It has been found [Hulme, unpublished data] that in young fruits (Bramley's Seedling variety), when detached from the tree, the soluble nitrogen rises but when "mature" fruits are stored a net protein synthesis takes place. It will be seen that in the fruits from uninjected trees (B.M. C (1)) the % of the total nitrogen in the form of protein has increased, i.e. a considerable net synthesis of protein has occurred in storage. On the other hand in the fruits from trees injected with ammonium nitrate the % protein-nitrogen has remained stationary, and there has been a slight decrease in this fraction in the fruit from the trees injected with asparagine. It remains for further work to decide whether this difference between the injected and control fruits is a direct one or whether the effect of the injection has been to retard the ripening of the fruits and so cause them to exhibit the characteristic behaviour of fruits not yet "mature". In this connexion it is important to learn the effect of the injection on the carbohydrate metabolism of the fruits. This is especially important in view of the recent work of Paech [1935] which suggests that in presence of excess soluble nitrogen compounds (ammonia and amides) protein synthesis may be limited by the amount of carbohydrate residues available.

SUMMARY.

1. Two varieties of apple trees were injected in the summer of 1935 with solutions of ammonium nitrate, urea and asparagine.

2. The fruit, gathered in the autumn, from the injected trees contained greater amounts of total nitrogen than from the uninjected trees; the distribution within the total nitrogen was also different.

3. When detached from the tree and placed at 9·5°, fruit from trees injected with ammonium nitrate and with asparagine did not synthesize protein, whereas that from untreated trees synthesized considerable amounts of protein.

4. Further experiments are necessary to determine whether this difference in behaviour is a direct result of the injection or whether it is due to delayed ripening of the fruits from the injected trees.

APPENDIX

A rapid continuous vacuum extractor.

The apparatus is shown in Fig. 2 which is drawn to scale. It is based on the Soxhlet principle operating under reduced pressure and in size is designed to handle up to 500 g. of frozen apple powder.

The flask, *A*, in which the solvent (600 ml. 85 % alcohol) is placed is connected to the extraction vessel, *B*, by means of a rubber sleeve. The side-tube, *S*, of the vessel *B* is wide (3 cm. diam.) and the bends in it are as "smooth" as possible to increase evaporation from the liquid in *A* by reducing frictional resistance to and turbulence in the vapour. The condenser, *C*, is a seven tube brass condenser[1] (seven ⅝ in. × 24 G. tubes 10 in. long in a jacket 2½ in. × 24 G.) at the top of which suction is applied by means of a water-jet pump. It is advisable to insert a calcium chloride trap between the pump and the condenser. For safety the author also inserts between the calcium chloride tower and the condenser a mushroom non-return valve actuated by gravity. The condenser is cooled by water which has passed through a large rectangular (30 ft. $\frac{3}{16}$ in. bore) copper coil immersed in an insulated ice-box.

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[1] This condenser was designed and made by Mr F. G. Forsyth of Ditton Laboratory.

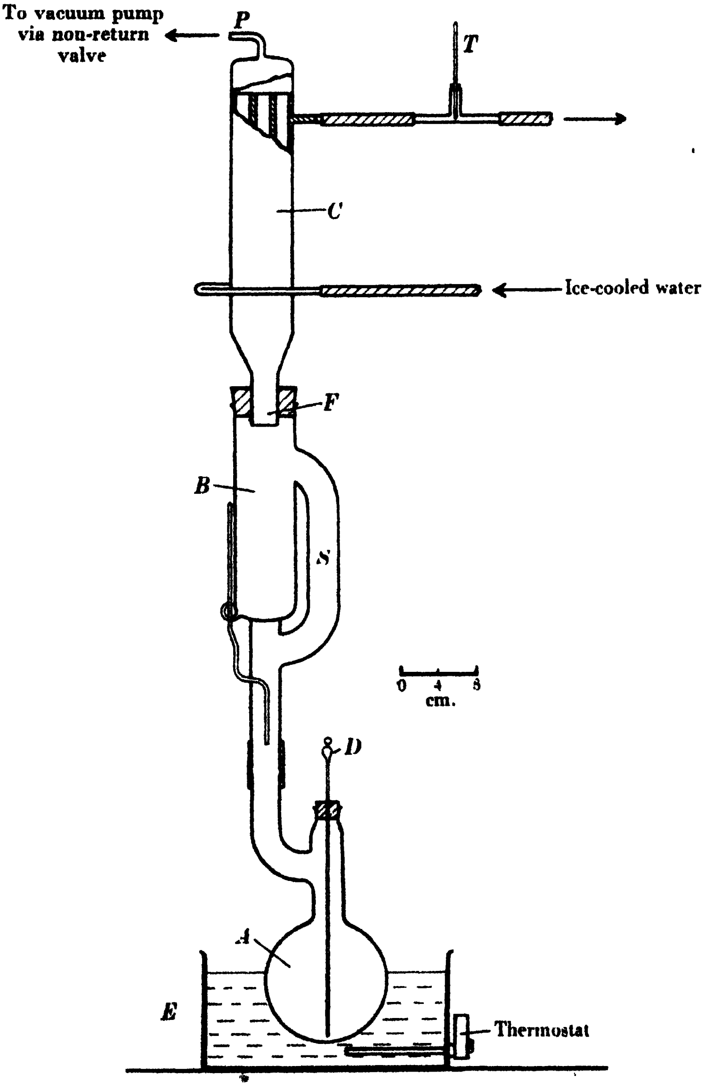

Fig. 2.

The thermometer, *T*, enables the rate of flow of the water through the condenser to be regulated to give maximum condensation. The bottom tube, *F*, of the condenser must be wide (3 cm.) to prevent bubbles forming where the rising vapour meets the falling condensate. Bubbler tube, *D*, is a Naumann stainless steel tube with a stiletto wire down the centre to allow of regulation of the rate of bubbling.

Water-bath, *E*, is thermostatically controlled to 45–50°. Table IV illustrates the "characteristics" of the extractor with 400 g. of apple pulp tissue in a thimble in vessel *B*. The figures are for one of two extractors running side by side in the same water-bath with the same suction line and condenser water supply.

Table IV.

Temperature of laboratory ° C.	Temperature of bath, *E* ° C.	Temperature of alcohol extract *B* ° C.	Temperature of thermometer *T* ° C.	Time for *B* to fill and siphon (min.)
16·9	45	20	10·3	6·5
18·4	49	20	9·5	4·5
18·6	47	21	10·0	5·0
19·0	47	21	10·0	5·5
19·2	48	22	9·4	5·0
19·5	45	22	9·8	6·0
20·0	50	22	10·0	4·5
21·0	48	22	9·7	4·8

The temperature of the liquid in the flask *A* never rises above 20° owing to the rapid evaporation from its surface. The concentration of the alcohol falling from the condenser into *B* is about 87 %.

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CXCIX. THE DECOMPOSITION OF ADENINE COMPOUNDS BY BACTERIA.

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(*Received 10 June 1936.*)

There seems to be at present good evidence that adenine compounds play an important role in muscle glycolysis and alcoholic fermentation. It would be of much biochemical interest to show whether they are also connected with the mechanism of bacterial fermentation. In order to approach this problem it was necessary first to study the ability of bacterial fermenters to attack adenine derivatives and to determine the content of adenosinetriphosphoric acid in their cells.

The following experiments were done to determine the exact conditions in which adenine and its derivatives undergo chemical changes by bacterial enzymes.

Experimental.

Organisms and culture. The experiments were first carried out with *Bact. coli*; they were subsequently repeated with other organisms (see p. 1409).

Unless otherwise stated the organisms were grown on tryptic broth agar in Roux bottles. After 20 hours' incubation the growth was washed off with distilled water and centrifuged and washed two or three times; the wet weight was then determined and the whole made up to a known volume in uniform suspension. The preparation was generally used the same day but could be kept 2 or 3 days at 0° without deteriorating. Sterilization of the culture by shaking with toluene and allowing to stand for 15 min. has no effect on the activity of the enzymes studied.

Reactions studied. The action of the bacterial suspensions in decomposing by deamination and/or dephosphorylation of the following substances was quantitatively investigated: adenosinetriphosphoric acid (adenylpyrophosphoric acid), muscle adenylic acid (adenine-9-furanoriboside-5-phosphoric acid), yeast adenylic acid (adenine-9-furanoriboside-3-phosphoric acid), adenosine and adenine; inosine and inosinic acid were also studied. Purified specimens of the compounds were used.

Technique. For each experiment the procedure was as follows: samples containing 1 ml. substrate, containing 0·085–0·120 mg. adenine-amino-nitrogen (NH_2–N) and 1 ml. bacterial suspension, corresponding to 30–160 mg. wet weight and 1 ml. water, which in some cases was replaced by salt solutions (phosphate, arsenate, fluoride, iodoacetate) were incubated at *p*H 7·0 in air and at 37°. It was however shown that all the reactions described below proceed as well anaerobically in nitrogen previously passed over heated copper. After a given time of incubation the samples were withdrawn from the incubator with corresponding blanks where the substrate was replaced by water, and 1 ml. of 20 % trichloroacetic acid was added.

The deamination was determined on the whole sample by the estimation of ammonia. It was carried out in the Parnas-Heller apparatus by distillation *in vacuo* with saturated borax solution and subsequent nesslerization [Parnas &

Heller, 1924]. The dephosphorylation was determined by phosphorus estimations in the trichloroacetic filtrate using the method of Fiske & Subbarow [1925] as modified by Lohmann & Jendrassik [1926].

Adenosinetriphosphoric acid (ATP). From Table I, 1 and 2, it is seen that in the course of 4 hours this substance is deaminated and dephosphorylated completely. It was found that small concentrations of magnesium (0·2 mg. Mg to 5 mg. ATP-barium salt) markedly increase the rate of breakdown of ATP; the appropriate quantity of $MgCl_2$ was accordingly used with this substance. In experiments (Table I, 3 and 4) where both deamination and dephosphorylation were estimated simultaneously in parallel samples, dephosphorylation appeared to precede deamination. This seems to be also the case with skeletal muscle enzymes [Mozolowski *et al.* 1932; Embden, 1933; Jacobsen, 1933] and excludes the possibility of the formation of inosinetriphosphoric acid. There is, however, an important difference in the behaviour of the enzymes of muscle and of *Bact. coli* towards ATP; in the former this substance is dephosphorylated by the elimination of the pyrophosphoric group only, forming adenylic acid, which is then deaminated to inosinic acid; in the latter case the bacterial enzymes attack not only the pyrophosphoric group but also the phosphorus atom of adenylic acid itself so that probably deamination does not occur till the stage when adenosine has been formed.

The decomposition is optimum at *p*H 7·2 and is inhibited at *p*H 5·5 and 8·8. Addition of phosphate slows down the breakdown of ATP, an effect which requires no special explanation as phosphate represents, in this case, a reaction product.

Table I.

	ATP containing				Deamination of ATP		Dephosphorylation of ATP				
No. exp.	Amino-N mg. N	Pyro-P (2P) mg. P	Total-P (3P) mg. P	Time of incubation *p*H 7·0 37°	Ammonia NH_3-N mg.	Of 1' amino-N appears as ammonia-N	Inorganic phosphate (P_0) mg. P	Phosphate after 7 min. hydrolysis in N HCl (P_7) mg. P	Pyrophosphate-P (expressed as P_7–P_0) mg. P	Of two atoms Pyro-P still unchanged	Of three atoms total-P appear as inorganic P
1	0·08	0·357	0·535	4 hr.	0·074	0·87	0·530	0·535	0·005	0	3
2	0·06	0·264	0·398	4 hr.	0·062	1	0·398	0·398	0·0	0	3
3	0·055	0·243	0·365	30 min.	0·003	0·05	0·004	0·233	0·229	1·87	0·03
	0·055	0·243	0·365	1 hr.	0·018	0·33	0·170	0·278	0·108	0·88	1·39
	0·055	0·243	0·365	1 hr. 30 min.	0·024	0·43	0·214	0·300	0·086	0·70	1·75
	0·055	0·243	0·365	4 hr.	0·052	0·95	0·365	0·365	0·0	0·0	3
4	0·060	0·264	0·398	30 min.	0·003	0·05	0·085	0·269	0·184	1·39	0·63
	0·060	0·264	0·398	1 hr.	0·010	0·16	0·114	0·296	0·182	1·39	0·86
	0·060	0·264	0·398	1 hr. 30 min.	0·025	0·42	0·169	0·307	0·138	1·04	1·28
	0·060	0·264	0·398	4 hr.	0·061	1	0·398	0·398	0·0	0	3

A remarkable effect of medium on enzyme production is noticeable in this connexion. When *Bact. coli* was grown on tryptic broth with 0·5 % glucose in a flask instead of on the surface of broth agar, the bacterial suspension when used in the same concentration as in previous experiments or even in twice that amount, repeatedly failed to attack ATP, whereas with other adenine compounds (with the exception of yeast adenylic acid to some extent, see below, p. 1407) no such effects were noticed. This suppression of enzyme formation was shown to be due to the presence of glucose, since it did not occur in cultures grown (1) in broth alone in strictly anaerobic conditions, or (2) in broth with 0·5 % formate. The suppression of the enzyme is, however, not complete as when 4–5 times the amount of bacteria was used (160 mg. instead of 30 mg. wet weight), positive results were obtained (Table II, 1 and 2). The partial suppression of the enzyme system may possibly be due to the acidity occurring during growth in glucose broth. This, however, does not account for the non-proportionality of the enzyme activity and bacterial concentration. This point needs further study.

Table II.

No. exp.	*Bact. coli* wet wt. mg.	Grown on	Time of incubation *p*H 7·0 37° hours	Deamination of ATP (containing 0·085 mg. NH_3-N)	
				Ammonia mg. N	% of deamination
1	40	Glucose broth	1	0·0	0
			2	0·0	0
			4	0·0	0
		Broth agar	1	0·013	15
			2	0·027	32
			4	0·072	85
2	50	Glucose broth	4	0·0	0
	80			0·0	0
	160			0·062	70

Muscle adenylic acid. This substance undergoes both deamination and dephosphorylation very rapidly, 2·5 mg. being decomposed by 10–20 mg. of bacteria (wet weight) in 40–60 min. No influence of small amounts of magnesium upon this reaction was found. Dephosphorylation evidently precedes deamination, as can be seen from Table III, 4 and 5. Bearing in mind that inosinic acid is readily dephosphorylated by *Bact. coli* (Table III, 3) and that, of all adenine compounds, adenosine is the most readily decomposed, not only by *Bact. coli* but also by other bacteria, one may suggest that deamination is conditioned by a preliminary dephosphorylation and occurs at the adenosine stage.

Table III.

No. exp.	Muscle adenylic acid containing		Yeast adenylic acid containing		Inosinic acid containing	Time of incubation *p*H 7·0 37°	Dephosphorylation expressed as		Deamination expressed as	
	P mg.	NH_2-N mg.	P mg.	NH_2-N mg.	P mg.		P mg.	%	NH_2-N mg.	%
1	0·21	0·095	—	—	—	2 hr. 30 min.	0·21	100	0·096	100
2	—	—	0·21	0·095	—	2 hr. 30 min.	0·20	95	0·082	85
3	—	—	—	—	0·18	2 hr. 30 min.	0·18	100	—	—
4	0·197	0·089	—	—	—	15 min.	0·144	73	0·025	26
	0·197	0·089	—	—	—	40 min.	0·168	85	0·060	67
	0·197	0·089	—	—	—	1 hr. 20 min.	0·170	86	0·080	89
	0·197	0·089	—	—	—	2 hr.	0·180	91	0·085	94
5	0·21	0·096	—	—	—	15 min.	0·164	78	0·040	41
	0·21	0·096	—	—	—	40 min.	0·188	89	0·070	73
	0·21	0·096	—	—	—	1 hr. 20 min.	0·192	91	0·084	87
	0·21	0·096	—	—	—	2 hr.	0·208	99	0·090	94

In the case of adenylic acid as well as in that of ATP, the addition of inorganic phosphate decreases the rate of breakdown. This time, however, a most marked effect can be produced by phosphoglyceric acid (3-phosphoglyceric acid) (Fig. 3) but not by hexosediphosphoric acid. Whether this is due to the Parnas-Ostern-Mann reaction [1934, 1, 2], i.e. a synthesis of ATP and a formation of pyruvic acid, will be investigated later. Pyruvic acid could of course not be detected owing to its rapid fermentation by *Bact. coli*.

Yeast adenylic acid. This is decomposed by bacterial enzymes both by dephosphorylation and deamination (Table III, 2). In experiments with deamination of both muscle and yeast adenylic acids, it has been repeatedly found that there is a difference in the rates of deamination of these two substances (Fig. 1). This effect is still more marked if the bacteria are grown on glucose broth (Fig. 2);

thus the presence of glucose in the growth medium affects this enzyme system in a manner similar to that described in the case of ATP, but to a much less marked extent.

It is well known that frog skeletal muscle enzymes are totally inactive towards yeast adenylic acid [Schmidt, 1928], whereas frog heart muscle enzymes act on it, a fact which suggested the existence of specific enzymes for the two substances [Ostern & Mann, 1933]. These experiments furnish further evidence for that possibility, a point to which we return later.

In this, as in all phosphorylated compounds tested, neither $M/60$ NaF nor $M/150$ iodoacetate had any effect.

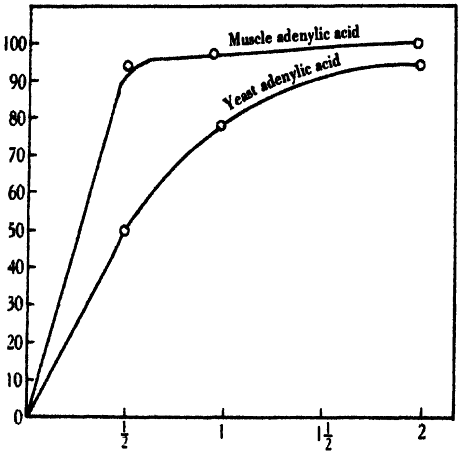


Fig. 1.

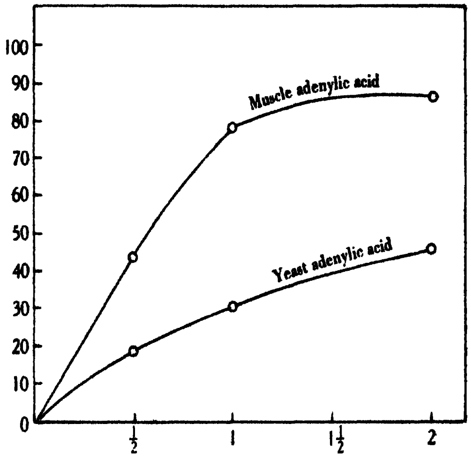


Fig. 2.

Fig. 1. Deamination of muscle adenylic acid and yeast adenylic acid by *Bact. coli*. grown on broth agar. (Average data from 9 experiments.) Abscissae: hours. Ordinates: % of deamination.

Fig. 2. Deamination of muscle adenylic acid and yeast adenylic acid by *Bact. coli* grown on glucose broth. (Average data from 7 experiments.) Abscissae: hours. Ordinates: % of deamination.

Adenosine. As well by *Bact. coli* as by any kind of bacteria studied in this work, adenosine is more rapidly decomposed than other adenine derivatives and than adenine itself. The optimum rate occurs at *p*H 8·8, whereas for all the other adenine derivatives it lies at *p*H 7·5. Small amounts of magnesium are without effect, but it is interesting to note that phosphate favours the deamination (Fig. 3). Phosphate can be replaced by arsenate.

Adenine. In this case the deamination depends largely on the presence of phosphate as in its absence the reaction—even on prolonged incubation—never exceeds 20 % of the total amino-nitrogen.

End-products. Hypoxanthine has been found and isolated as picrate of the silver salt ($C_5H_3N_4AgO$, $C_6H_2(NO_2)_3OH$) in the kieselguhr filtrate obtained from incubated samples of the following substances with the usual amount of bacterial suspension: adenosine, adenine, inosine, inosinic acid. The picrate was obtained from samples corresponding to about 5 mg. of hypoxanthine according to the procedure of Bruhns [1890] and identified by silver estimation (23 % Ag, theor. 22·88 %). It was also possible owing to the kind help of Dr M. Dixon to show the presence of hypoxanthine in a biological way using a highly specific preparation of xanthine oxidase [Green & Dixon, 1934; Dixon & Lemberg, 1934].

At the stage at which hypoxanthine is formed, no ribose could be traced by the orcinol test.

Comparison of bacteria studied. In order to get information about the distribution of the group of enzymes concerned, the following organisms were studied: *Bact. dispar*, *Bact. cloacae*, *Bact. lactis aerogenes*, *Str. faecalis*, *Ps. pyocyanea*. The results are summarized in Table IV, where it can be seen that *Bact. dispar*, *Bact. cloacae* and *Bact. lactis aerogenes* show a behaviour very similar to that described for *Bact. coli*. If *Bact. lactis aerogenes* is grown on glucose broth instead of on agar, there occurs the same effect of depressing the enzyme dealing with ATP and yeast adenylic acid as described for *Bact. coli*, but in a slighter degree, probably owing to the fact that its fermentation products in the culture medium are less acid than those of *Bact. coli*. *Str. faecalis* even if used in large amounts shows very

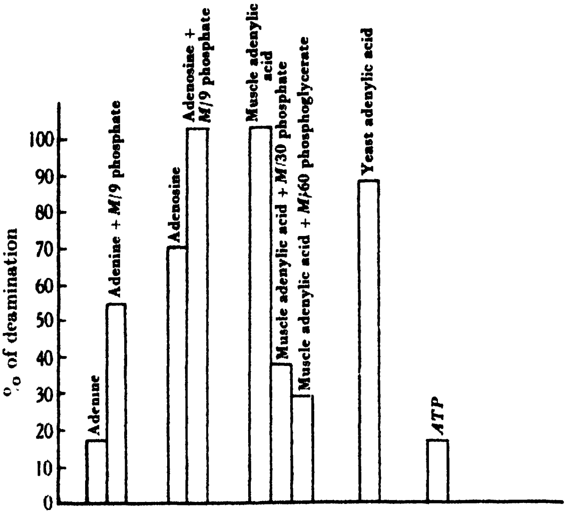


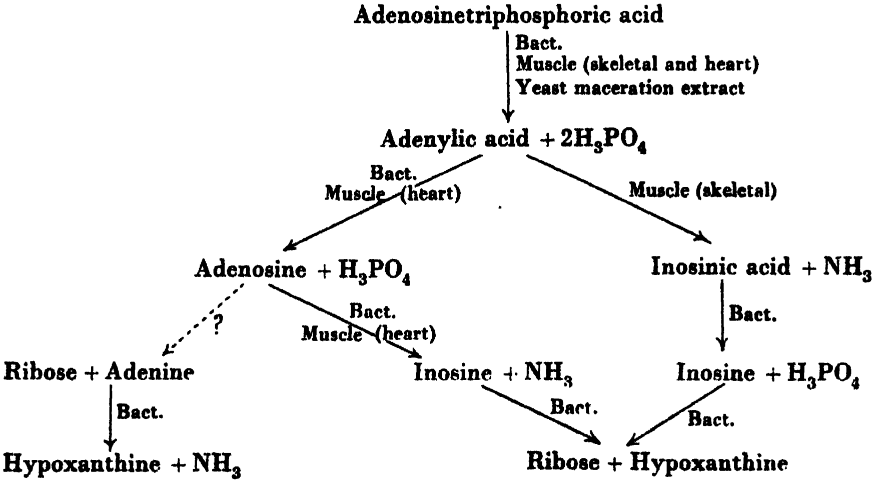
Fig. 3. Deamination of adenine compounds by *Bact. coli* after 1 hour of incubation.

Table IV.

No. exp.	Organism	Time of incubation pH 7·0 37° hr.	Deamination of adenine compounds: ATP		Muscle adenylic acid		Yeast adenylic acid		Adenosine		Adenine	
			NH_2-N mg.	% of added	NH_2-N mg.	% of added	NH_2-N mg.	% of added	NH_2-N mg.	% of added	NH_2-N mg.	% of added
1	*Bact. dispar.* 30 mg. wet weight; grown on broth agar	1	0·0	0	0·050	52	0·027	28	0·092	76	0·010	8
		2	0·0	0	0·080	83	0·055	57	0·112	93	0·030	25
		4	0·018	21	0·096	100	0·090	94	0·120	100	0·060	51
2	*Bact. cloacae.* 30 mg. wet weight; grown on broth agar	1	0·012	14	0·079	82	0·036	38	0·104	86	0·041	34
		2	0·028	29	0·090	93	0·065	67	0·112	93	0·065	54
		4	0·057	58	0·097	100	0·085	88	0·120	100	0·095	79
3	*Bact. lactis aerogenes.* 30 mg. wet weight; grown on broth agar	1	0·080	94	0·090	94	0·080	83	0·105	87	0·060	50
		2	0·083	98	0·090	94	0·083	86	0·110	92	0·068	66
		4	0·086	100	0·095	100	0·092	95	0·120	100	0·100	83
	grown on glucose broth	2	0·023	27	0·070	72	0·040	42	—	—	—	—
4	*Str. faecalis.* 100 mg. wet weight; grown on glucose broth	4	0·010	11	0·020	20	0·020	20	0·020	18	0·020	18
5	*Ps. pyocyanea.* 30 mg. wet weight; grown on broth agar	1	0·010	11	0·010	10	0·015	15	0·060	50	0·060	47
		2	0·015	18	0·020	20	0·015	15	0·115	96	0·105	82

low values. *Ps. pyocyanea* has been found to possess active deaminases but it evidently lacks dephosphorylating enzymes. The data in Table IV show clearly the difference already mentioned in the rates of deamination of muscle and yeast adenylic acids, giving again evidence for specificity of both enzymes.

Scheme. The following scheme has been made to compare the results obtained with those already known for frog skeletal and heart muscles and also for yeast maceration extract.



The content of adenosinetriphosphate in Bact. coli.

Bact. coli were grown on tryptic broth agar for 20 hours in 10 Roux bottles. The cultures were washed twice and centrifuged and weighed. The average wet weight of the cultures from 10 Roux bottles after 20 hours' growth was 10 g. with the average water content of 80 %. The total crop was then suspended in 20 ml. of water and treated in one of two ways. (1) It was heated for 20 min. in a boiling water-bath and then precipitated with trichloroacetic acid and left for 10 hours in ice (Table V, 1, 2, 6). (2) It was ground for 30 min. with very sharp quartz sand (Merck) and trichloroacetic acid in a porcelain mortar, then thoroughly shaken and allowed to stand for 10 hours in ice after which it was again ground (Table V, 3, 4, 5). In both cases the final concentration of trichloroacetic acid was 7 %. The trichloroacetic precipitate was removed either by filtration or by centrifuging and a clear yellowish solution resulted. From this ATP was isolated as barium salt and estimated by deamination following the procedure given by Parnas & Lutwak-Mann [1935].

A known volume of the trichloroacetic extract was placed in a centrifuge-tube; it was made alkaline with 10 % NaOH to *p*H 8·0, treated with barium acetate 50 % and kept for 30 min. at 0°. The barium precipitate was centrifuged and washed twice with 1 % barium acetate, whilst the supernatant fluid tested with barium acetate was poured off and prepared for the estimation of adenylic acid as will be described below (B). The barium precipitate containing among other substances the total ATP—if present—was completely dissolved in hydrochloric acid, the barium removed with Na_2SO_4, the solution neutralized and made up to a known volume (A). The solution B which might contain adenylic acid was freed from barium and ammonia, neutralized and made up to a known volume. Both solutions A and B were next divided into two parts, one was used as a blank, the other one treated with preparation of specific deaminase from frog skeletal muscle [see Parnas & Lutwak-Mann, 1935]. All samples were incubated at 37° for 4 hours. Ammonia estimation followed. In some cases a frog heart muscle preparation was used but with no difference in result. It was, of course, impossible to use a suspension of *Bact. coli*, as this enzyme preparation, unlike muscle, contains a great number of different deaminases.

A rather low value (Table V) has been found for the ATP content in *Bact. coli* if compared with that in muscle or yeast [Lohmann, 1928, 1, 2; Lutwak-Mann & Mann, 1935], about 0·7–1·5 mg./100 g. (wet weight) amino-nitrogen and 4 mg./100 g. P as pyrophosphoric group, as was estimated by Lohmann's hydrolysis method [1928, 1], but a good agreement existed between data from different experiments. For the adenylic acid, however, negative results were obtained, which agrees with findings for muscle tissue [Lohmann & Schuster, 1934; Parnas & Lutwak-Mann, 1935].

Table V.

Content of ATP in *Bact. coli*

No. exp.	Wet wt. of bacteria g.	NH_2-N of ATP in the total mg. N	NH_2-N of ATP in 100 g. of bacteria (wet wt.) mg. N
1	10·00	0·150	1·50
2	10·64	0·078	0·73
3	15·60	0·183	1·17
4	9·40	0·098	1·04
5	10·00	0·102	1·02
6	11·70	0·134	1·14

Other purine compounds. A few experiments only were carried out on guanine compounds with the result that guanylic acid was found to be dephosphorylated and guanosine deaminated in the presence of phosphate by *Bact. coli*.

SUMMARY.

1. Washed suspensions of *Bact. coli* and other members of the Bacteriaceae decompose the following adenine compounds: adenosinetriphosphoric acid, muscle adenylic acid, yeast adenylic acid, adenosine, adenine, the first three being both dephosphorylated and deaminated, the latter two being deaminated.
2. The reactions occur either aerobically or anaerobically and in fresh suspensions as well as in those treated with toluene.
3. The end-product of decomposition is hypoxanthine. At this stage ribose is absent.
4. Adenosinetriphosphate (but not adenylic acid) is found to be a cell constituent of *Bact. coli*.

I am glad to take this opportunity of expressing my deepest gratitude to Dr Marjory Stephenson, whose encouragement and advice were invaluable. My thanks are also due to Sir F. G. Hopkins for his kind interest in this work.

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CC. THE HEAT COAGULATION OF CASEINOGEN.

II. THE RATE OF PHOSPHORUS CLEAVAGE.

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In a previous paper [Howat & Wright, 1934] it was shown that drastic heat treatment of neutral solutions of calcium caseinogenate resulted in a gradual dephosphorylation of the caseinogen molecule and ultimately in coagulation of the protein. There was also a slow liberation of acid-soluble nitrogen.

In the original experiments the protein solutions were heated in closed tubes at 120°. It appeared desirable to carry through a further series of experiments using a lower range of temperatures. It was hoped by this means (*a*) to determine the temperature coefficients of the reactions, and (*b*) to secure further evidence regarding the relationship between phosphorus cleavage and the coagulation of the protein.

Technique.

Solutions of calcium caseinogenate were prepared by the technique described in the earlier paper, but were made up to contain 3·0 % in place of 3·5 % caseinogen. The amount of $Ca(OH)_2$ used was 0·065 g. per 100 ml., which gave a *p*H of roughly 6·9. 5 ml. portions of the solution were heated in closed tubes in a thermostatically controlled glycerol bath. The temperatures employed ranged from 90 to 115°. The periods of heating varied from 1 hour up to 45 hours, the time necessary to produce dephosphorylation and coagulation of the protein being found to increase progressively as the temperature of heating was lowered.

At suitable time intervals duplicate tubes were removed from the heating bath. One tube was used for estimating acid-soluble phosphorus and nitrogen and the second tube for determining the extent of protein coagulation. The latter figure was ascertained by centrifuging the solution for 5 min. at 3000 r.p.m. and determining the percentage of uncoagulated protein in the supernatant liquid.

Results.

The results are shown in Figs. 1–5. It will be seen that at all the temperatures employed the curves show a close similarity to those obtained in the previous work. There is a slow liberation of acid-soluble nitrogen and a relatively rapid liberation of acid-soluble phosphorus. The proportion of phosphorus liberated does not exceed 60 %. As regards coagulation of the protein, this takes place slowly at first, and then more rapidly. A final slowing-up of the reaction gives a typical "S" shape to the curve. It may be noted that visible coagulation usually occurs when 50–60 % of the protein has been rendered insoluble, namely on the steep gradient of the curve.

In order to determine the temperature coefficients of dephosphorylation and heat coagulation, a diagram has been constructed (Fig. 6) in which the temperature of heating is plotted against the time of heating (expressed logarithmically

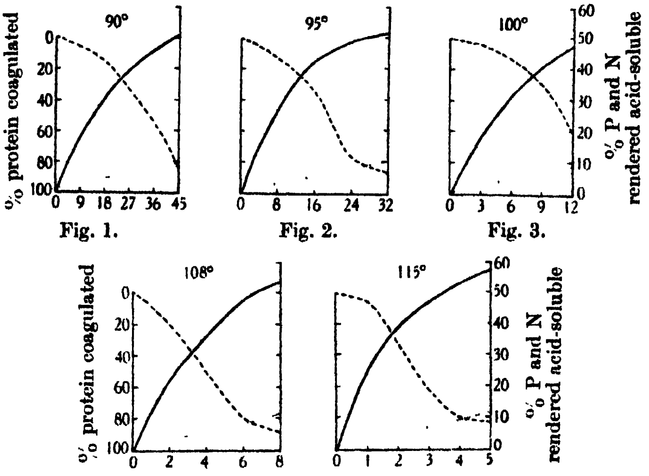

Fig. 1. Fig. 2. Fig. 3.

Fig. 4. Fig. 5.

Figs. 1 to 5. Effect of various temperatures of heating. (Continuous line, acid-soluble phosphorus; dotted line, acid-soluble nitrogen; broken line, protein coagulated. Time in hours shown below each figure.)

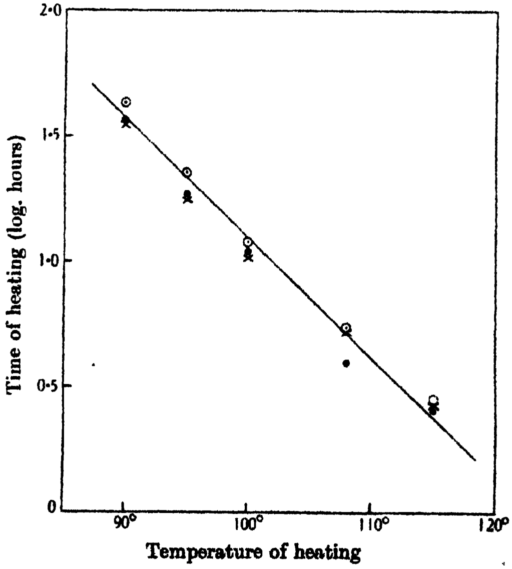

Fig. 6. Time-temperature relationships. (White circles, visible coagulation; black circles, 50% protein coagulated; crosses, 45% dephosphorylation.)

in hours) required to produce (*a*) 45% dephosphorylation, (*b*) visible coagulation and (*c*) 50% coagulation of the protein. It will be seen that all the points fall roughly on a straight line. This fact provides strong presumptive evidence that dephosphorylation and heat coagulation are related reactions, coagulation probably being preceded by liberation of phosphorus from the caseinogen molecule.

It may be noted that the slope of the curve shows that, for each rise in temperature of 10°, the reaction velocity increases threefold. This fact has one interesting application. For various reasons it is necessary to store milk products, such as evaporated milk, for relatively long periods. It appeared possible that during such prolonged storage dephosphorylation of the caseinogen might occur, with consequent coagulation of the protein. In order to obtain some idea of the rate of dephosphorylation and coagulation at storage temperatures the curve in Fig. 6 was extrapolated to 20°. It was found that at this temperature dephosphorylation would take well over 7 years—a storage period completely outside the range of commercial practice. To confirm this point samples of caseinogen were prepared from freshly manufactured evaporated milk and from evaporated milk which had been stored for 6–9 months. The N/P ratios of the caseinogen were found to be as follows:

Freshly manufactured samples	18·91, 21·31, 21·32, 19·62	Mean 20·29
Stored samples	19·14, 21·20, 20·90, 20·90, 18·50, 19·90	Mean 20·09

These figures show that during 6–9 months' storage at ordinary temperatures there was no detectable liberation of phosphorus from the caseinogen molecule.

SUMMARY.

1. A study has been made of the rates of liberation of nitrogen and phosphorus from neutral solutions of calcium caseinogenate at temperatures between 90 and 115°. Determinations of the rate of coagulation of the protein have been made simultaneously.

2. The results confirm the conclusions reached in previous work, namely that the dephosphorylation and heat coagulation of caseinogen are related reactions.

3. It is shown that for each rise in temperature of 10° the reaction velocity of dephosphorylation and coagulation increases three-fold.

4. No detectable liberation of phosphorus takes place as a result of storing evaporated milk at ordinary temperatures for periods of 6–9 months.

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CCI. THE NON-PROTEIN-NITROGEN OF PULSES.

II. PARTITIONING OF THE NITROGEN AND A DETERMINATION OF THE ESSENTIAL AMINO-ACIDS.

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(*Received 10 May 1936.*)

In a previous communication [1935] attention was drawn to the presence of an easily assimilable non-protein nitrogenous fraction occurring in the pulses to the extent of 10–55 % of the total nitrogen and consisting mostly of the simpler polypeptides. The present study relates to an investigation of the amino-acid make-up of the fraction from the three well-known pulses, *Phaseolus aconitifolius*, *Cicer arietinium* and *Phaseolus mungo*.

Experimental.

Preliminary trials with *P. aconitifolius* showed that 0·5 % acetic acid was the best solvent, yielding the maximum amount of the non-protein-nitrogen. It was found that a 5 % solution of sodium chloride, whilst extracting the globulins and albumins of the seed, does not effectively dissolve out the non-protein-nitrogen; further, the sodium chloride which persists in the non-protein fraction after the removal of proteins by heat coagulation or acid precipitation cannot be easily eliminated. 0·5 % acetic acid, on the other hand, is not only an efficient solvent for the extraction of the non-protein-nitrogen but can be easily distilled off. It has the further advantage of effecting a coagulation of the globulins during the process of extraction itself.

With a view to determining the percentage of the non-protein-nitrogen (N.P.N.) present in the seed meal, the material (10 g.) was repeatedly (3–5 times) extracted with 100 ml. portions of dilute acetic acid in the cold at 0° to eliminate the possibility of proteoclastic action. Successive extracts obtained by centrifuging the mash were kept separately, warmed up to 60° to coagulate any albumins that might be present, treated with finely disintegrated Seitz asbestos, filtered and the resulting clear filtrate made up to a known volume, an aliquot of which was employed for a determination of the total nitrogen (T.N.). The clear filtrates were found to yield no precipitate or any opalescence on the addition of trichloro-acetic acid, showing thereby that all the proteins had been eliminated. In the case of two pulses, *C. arietinium* and *P. mungo*, filtrations of the later extractions became increasingly difficult and yielded turbid filtrates, owing to the simultaneous extraction of increasing amounts of starch whose presence could be detected by the iodine test.

It will be observed from Table I that *P. aconitifolius* has a non-protein-nitrogen content which is about thrice that of *C. arietinium* and about four times that of *P. mungo*. The first extraction removes 54–68 % of the N.P.N. present in the seed whilst the yield and purity of the subsequent extracts are low, owing to the extraction of increasing concentrations of carbohydrates whose elimination is

Table I.

Pulse	T.N. as % on the wt. of moisture-free seed	% of N.P.N. on the T.N.	% of N.P.N. obtained at each of the extractions 1	2	3	4	5
P. aconitifolius	3·85	34·85	62·62	21·88	8·42	4·32	2·75
C. arietinium	3·50	11·63	68·01	16·55	8·82	6·62	—
P. mungo	3·74	8·86	54·63	22·69	22·69 (?)	—	—

extremely difficult and whose presence seriously interferes with the analysis of the N.P.N. In our present study, therefore, we have confined our attention to the investigation of the first extract, for obtaining a large quantity of which the following procedure was adopted.

The seed meal (1 kg.) was extracted for 24 hours at 0° with 10 litres of 0·5 % acetic acid. The extract after filtration was warmed to 60°, treated with asbestos and again filtered, a clear protein-free filtrate being thus obtained. The filtrate was concentrated at 45–50° under reduced pressure to about 500 ml., and was then preserved in the ice-chest for subsequent experiments.

A partitioning of the nitrogen by the method of Van Slyke as modified by Damodaran [1931] so as to include the dicarboxylic acid-nitrogen, and an independent estimation of some of the essential amino-acids have been carried out. Arginine was determined by the arginase-urease method of Hunter & Dauphinee [1930] whilst the colorimetric methods of Folin & Marenzi [1929] were adopted with minor modifications for the estimation of tyrosine, tryptophan and cystine. During the estimation of tryptophan, the precipitate of mercury-tryptophan complex was decomposed by H_2S yielding a light-coloured filtrate which was satisfactory for the colorimetric estimation. In the cystine estimation the precipitation of the mercury compound was carried out at pH 4·6, at which the cystine was quantitatively precipitated as revealed by preliminary experiments with known additions of cystine to the experimental hydrolysate. The mercury compound was suspended in dilute H_2SO_4 solution and was decomposed by H_2S. HgS was removed by filtration and the excess of H_2S from the filtrate eliminated by passing in a current of CO_2. For the estimation of tyrosine, no special modification in procedure was found to be necessary. Tyrosine and tryptophan were determined on 5 ml. of the non-protein-nitrogen concentrate, which were hydrolysed with 5 ml. of a 20 % alkali for 18 hours on a boiling water-bath, whilst for cystine determination, 10 ml. of the material were treated with a sufficient amount of sulphuric acid so that the resulting concentration of the acid in the mixture was 4 %. The hydrolysis was allowed to proceed for 3 hours in a metal-bath maintained at 110°. For Van Slyke analysis, however, the hydrolysis was carried out under the same conditions of temperature, time and acid concentration except for the fact that hydrochloric in place of the sulphuric acid was employed. Preliminary trials showed that under these conditions a complete fission of the peptide linkages had occurred with the formation of the minimum amount of humin.

Table II. *Van Slyke partitioning of the nitrogen as percentages of the total.*

Form of nitrogen	Melanin	Amide	Dicarboxylic	Basic amino	Basic non-amino	Non-basic amino	Non-basic non-amino	Total
P. aconitifolius	1·24	5·54	23·72	20·56	24·98	25·76	0·51	102·31
C. arietinium	2·34	6·11	13·06	15·22	44·29	19·31	0·04	100·39
P. mungo	2·37	3·41	16·16	11·21	32·46	19·95	16·52	102·10

A study of the above results (Table II) reveals that the N.P.N. fraction of aconite bean has a higher percentage of dicarboxylic acid nitrogen than either of the other two fractions, whilst *C. arietinium* contains the maximum amount of the basic nitrogen. The proline-hydroxyproline content of *P. mungo* appears to be exceptionally high as revealed by the non-amino-nitrogen in the non-basic fraction.

Table III. *Essential amino-acids as percentages of total nitrogen.*

	Arginine (Van Slyke)	Arginine (enzyme)	Tyrosine	Tryptophan	Cystine
P. aconitifolius	12·71	11·85	0·84	0·42	0·22
C. arietinium	45·48	22·92	0·60	0·50	1·13
P. mungo	34·59	16·30	0·78	1·15	3·39

Table III shows that whilst in the case of *P. aconitifolius* there is a fair agreement between the arginine values obtained by the Van Slyke and enzyme methods, there is a great discrepancy between similar pairs of values for the other two pulses, the values by the enzyme method being about half those by the Van Slyke method. This discrepancy points to the existence of bases other than arginine which are capable of yielding ammonia during alkali hydrolysis and which appear to possess no free amino-group capable of reacting with nitrous acid, as revealed by the high values for non-amino-nitrogen (Table II) in the basic fraction. Further work to characterise these bases is now in progress.

The percentage of tyrosine in the non-protein-nitrogen fraction is very much lower than the tyrosine content of globulins associated with the respective seeds, as given by Niyogi *et al.* [1932]. The tryptophan content in general follows the same order as obtains in the globulins, whilst the cystine content of the fraction from *P. mungo* is very much higher than the cystine content of the globulins of the seed as reported by Niyogi *et al.* [1932].

SUMMARY.

1. The non-protein-nitrogen fractions of the three pulses, *P. aconitifolius*, *C. arietinium* and *P. mungo*, have been extracted by dilute acetic acid and concentrates prepared for analysis. The nitrogen of the fraction has been partitioned by the method of Van Slyke as modified by Damodaran to include dicarboxylic acid-nitrogen.

2. An independent determination of the essential amino-acids, tyrosine, tryptophan, cystine and arginine by the arginase-urease method has been carried out. The results strongly point to the existence of a new base other than arginine.

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CCII. VITAMIN C REQUIREMENTS OF THE GUINEA-PIG.

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Van Eekelen *et al.* [1933], Hess & Benjamin [1934] and Johnson & Zilva [1934] have shown that human beings do not excrete ascorbic acid or excrete it only to a limited extent when it is ingested by them unless they are fully "saturated" with the vitamin. The last-named workers were at the same time able to show by means of detailed quantitative experiments that certain individuals may be so depleted of the vitamin as to take a considerable time to reach the condition of saturation when consuming daily doses of 100–150 mg. of vitamin C. Yet such individuals not only fail to display any symptoms even of latent scurvy but appear to be in perfect bodily and mental vigour. Similar results have since been obtained by a number of workers. Some of these are, however, inclined to attribute to this lack of vitamin C "saturation" a clinical significance which in the opinion of the writer is not warranted by the existing knowledge of the subject. Experiments on guinea-pigs are given below which throw some further light on the matter.

The chief aim of this investigation was to ascertain the relationship of the intake of ascorbic acid to the vitamin C content of the "selective" organs and other tissues of these animals, to their susceptibility to scurvy, to the urinary excretion of ascorbic acid and to their general well-being.

Storage of vitamin C and susceptibility to scurvy.

That guinea-pigs of about 300 g. maintained on a good diet with cabbage *ad lib.* under favourable hygienic conditions show little individual variation in susceptibility to the disease is well known to workers in experimental scurvy. The tissues of these animals, especially the "selective" tissues such as the adrenals, anterior lobe of the pituitary, the liver, the small intestine etc. contain vitamin C in quantities which cannot be raised even by further administration of high doses of ascorbic acid either *per os* or by injection. In other words, when the tissues of growing guinea-pigs are supplied with vitamin C to their maximum capacity, the animals vary little in the time taken by them to succumb to scurvy.

The following experiments were designed in order to obtain more detailed knowledge concerning the influence of the bodily store of vitamin C in the guinea-pig on the time of survival on a scorbutic diet. For this purpose chosen animals, as used for customary vitamin C testing, were placed on the scorbutic diet employed in this laboratory. During the first 10 days the various groups received *per os* daily doses of 0·5, 1, 2, 3, 10 and 20 mg. of ascorbic acid respectively. At the end of this time the daily dose was discontinued and the animals were allowed to remain on the scorbutic diet until they succumbed. It is seen from Fig. 1 that the time taken by group 1 (daily dose 0·5 mg.) to succumb to scurvy after receiving the last dose of ascorbic acid (average time about 20 days) was

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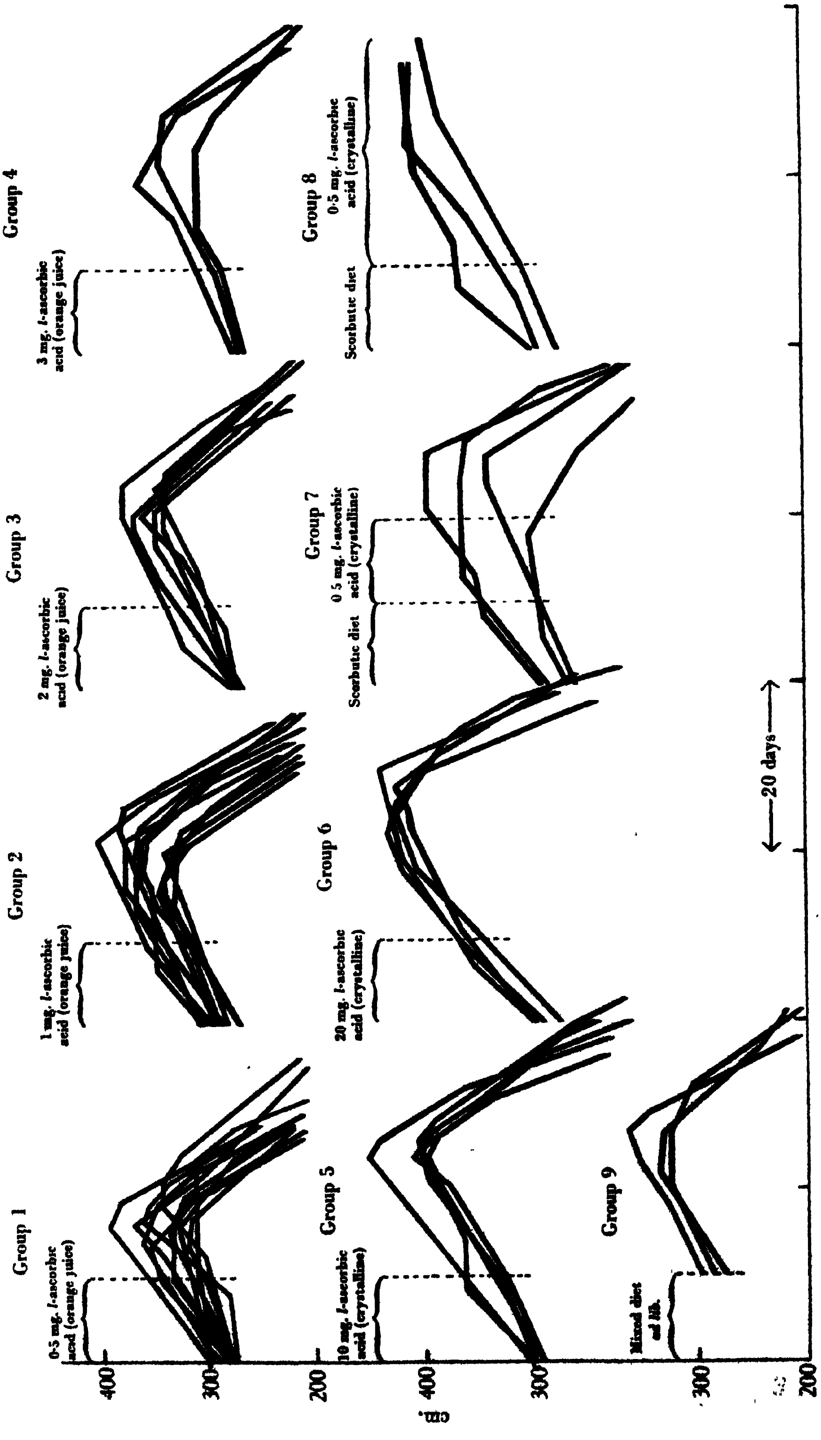

Fig. 1. All the animals except those in group 8 died of scurvy, uncomplicated by any other disease.

less than that taken by the control group 9. The animals in group 2 also died of the deficiency earlier than the control group of animals, but the difference here is less marked than in the case of group 1. The remaining groups appear to the writer not to have shown any significant deviation in their behaviour on the deficient diet from that of the animals which consumed very generous supplies of vitamin C in the form of cabbage during the period preceding the deprivation of the vitamin. It is of interest to note that when the guinea-pigs were depleted of vitamin C previous to the period of dosage with 0·5 mg. of ascorbic acid the time taken by the animals to succumb to scurvy (group 7) was almost the same as when no previous depletion took place (group 1). Furthermore, a dose of 0·5 mg. administered daily after exhausting the animals of their vitamin C store by maintaining them on a scorbutic diet for 10 days was sufficient to prevent the production of macroscopic lesions after 37 days when the animals were killed by chloroform (group 8). The import of these results will receive comment in a subsequent section when they will be considered in conjunction with the other information obtained in this investigation.

The influence of the quantity of ascorbic acid consumed on the concentration of the vitamin in the tissues.

The original aim was to establish the minimum daily dose of ascorbic acid necessary to be consumed *per os* by the guinea-pig in order that the animal should excrete it in appreciable amounts in the urine and to correlate this dose with the concentration of the vitamin in the tissues. This could not be ascertained by direct experiment, since it was found that the guinea-pig had to consume extremely high doses of ascorbic acid before any appeared in the urine. Recourse was, therefore, made to injection after which, as has been shown already by the writer [Zilva, 1935, 1, 2], urinary excretion of the vitamin quickly follows. Analyses of the tissues of a number of guinea-pigs which received single graded injections were therefore carried out and compared with those of groups of animals receiving similar doses *per os*.

All the injections, unless otherwise stated, were performed intramuscularly (femoral muscle) without an anaesthetic. The injected animals were previously depleted of vitamin C by maintenance on a scorbutic diet for 6 days but the other groups received their dose orally for 15 days whilst subsisting on a scorbutic diet without being previously depleted. Details of the method of analysis have already been described in a previous communication [Zilva, 1935, 1].

It is seen from the figures in Table I that, considering the limitations of the method, the injection of a single dose of ascorbic acid brings about in the tissues of the guinea-pig a concentration of the vitamin similar to that found when the animal receives orally an equal dose daily for 15 days whilst on a scorbutic diet. Consequently the necessary concentration of ascorbic acid in the tissues following injection which permits its excretion in the urine can be reached by the same dose given *per os* for 15 days. It is further seen that a dose of 5–8 mg. was required by the guinea-pig on a scorbutic diet before even a trace of the vitamin could be detected in their tissues and finally that the highest concentration approximately equal to that obtained by the injection of 50 mg. was only attained when doses of about 20 mg. were employed.

Results obtained by Jacobsen [1935], Giroud *et al.* [1935], Fox & Levy [1936] and De Caro [1936], in connexion with the deposition of ascorbic acid in the tissues agree in principle with those given above. Owing, however, to the different experimental procedures adopted by these workers, especially by the former, direct comparison of the quantitative data is made difficult.

Table I.

	Wt. of animal g.	Scorbutic diet days	Method of administration	Quantity found										Excreted in urine mg.	Gain in wt. g.
				Small intestine		Large intestine		Liver			Carcass				
				mg./g.	Total mg.	mg./g.	Total mg.	mg./g.	Total mg.	Adrenal mg./g.	mg./g.	Total mg.	Pituitary		
				3 mg. *l*-ascorbic acid.											
	270	6	Intramuscular injection	0·05	0·6	0·03	0·3	0·06	0·7	0·1	0·01	1·3	0 to trace	0	10
	280	6	,,	0·04	0·5	0·02	0·3	0·06	0·5	0·0	0·00	0·0	,,	0	20
	277	6	,,	0·06	0·9	0·05	0·5	0·05	0·6	0·1	0·01	1·8	0	0	17
	282	6	,,	0·07	0·8	0·02	0·2	0·06	0·7	0·0	0·01	1·0	0 to trace	0	22
Average	277	6	—	0·06	0·7	0·04	0·3	0·06	0·6	0·1	0·01	1·0	—	0	17
				3 mg. *l*-ascorbic acid daily.											
	350	15	*Per os*	0·08	1·2	0·06	0·7	0·05	0·8	0·0	0·10	2·3	0	—	70
	295	15	,,	0·05	0·7	0·04	0·5	0·06	0·7	0·2	0·01	0·9	Trace	—	25
	310	15	,,	0·06	0·7	0·04	0·5	0·05	0·6	0·0	0·00	0·0	+	—	45
	305	15	,,	0·05	0·7	0·04	0·4	0·05	0·5	0·0	0·00	0·5	0	—	40
Average	315	15	—	0·06	0·8	0·05	0·5	0·05	0·7	0·1	0·01	0·9	—	—	45
				5 mg. *l*-ascorbic acid.											
	290	6	Intramuscular injection	0·05	0·6	0·05	0·5	0·14	1·5	0·0	0·00	0·0	0	0	25
	290	6	,,	0·05	0·6	0·05	0·5	0·05	0·6	0·1	0·01	2·0	Trace	0	5
	275	6	,,	0·07	1·1	0·07	0·8	0·09	0·9	0·2	0·01	1·7	0 to trace	0	10
	285	6	,,	0·10	1·2	0·05	0·5	0·08	0·9	0·2	0·01	2·4	+ to + +	0	18
Average	285	6	—	0·07	0·9	0·06	0·6	0·09	1·0	0·1	0·01	1·5	—	0	15
				5 mg. *l*-ascorbic daily.											
	315	15	*Per os*	0·08	1·2	0·05	0·6	0·05	0·6	0·0	0·01	1·6	Trace to +	—	50
	335	15	,,	0·06	0·9	0·05	0·5	0·03	0·5	0·0	0·01	1·2	+	—	70
	310	15	,,	0·07	1·0	0·04	0·4	0·05	0·6	0·3	0·01	2·1	+ +	—	45
Average	320	15	—	0·07	1·0	0·05	0·5	0·04	0·6	0·1	0·01	1·6	—	—	55

8 mg. *l*-ascorbic acid.															
	300	6	Intramuscular injection	0·10	1·1	0·06	0·7	0·12	1·5	0·4	0·02	2·6	+ + +	0·0	30
	295	6	,,	0·07	0·9	0·05	0·5	0·06	0·6	0·2	0·02	2·6	+ + + +	0·1	35
	285	6	,,	0·10	1·1	0·07	0·6	0·08	1·1	0·1	0·02	2·4	+ to + +	0·0	25
	272	6	,,	0·10	1·1	0·08	0·7	0·10	1·0	0·4	0·02	3·0	+ + to + + +	0·1	7
Average	288	6	—	0·09	1·1	0·07	0·6	0·09	1·1	0·3	0·02	2·7	—	0·1	24
8 mg. *l*-ascorbic acid daily.															
	347	15	*Per os*	0·06	0·7	0·04	0·5	0·05	0·7	0·1	0·01	2·2	Trace to +	—	67
	355	15	,,	0·06	0·9	0·05	0·6	0·04	0·6	0·1	0·01	2·3	+ to + +	—	75
	360	15	,,	0·08	1·1	0·06	0·7	0·07	1·0	0·0	0·02	3·8	Trace to +	—	60
	325	15	,,	0·07	1·0	0·05	0·6	0·07	1·0	0·6	0·02	2·8	Trace	—	25
Average	346	15	—	0·07	0·9	0·05	0·6	0·06	0·8	0·2	0·02	2·8		—	57
10 mg. *l*-ascorbic acid.															
	292	6	Intraperitoneal injection	0·08	1·1	0·05	0·5	0·07	0·8	0·3	0·02	3·3	0 to trace	0	32
	290	6	,,	0·10	1·2	0·09	0·9	0·08	1·1	0·0	0·03	4·8	Trace	0	25
Average	291	6	—	0·09	1·2	0·07	0·7	0·08	1·0	0·2	0·03	4·1		···	28
10 mg. *l*-ascorbic acid daily.															
	300	15	*Per os*	0·10	1·2	0·05	0·6	0·08	1·2	0·5	0·02	3·1	+	—	25
	330	15	,,	0·09	1·2	0·05	0·6	0·05	0·5	0·4	0·01	2·2	+	—	35
	330	15	,,	0·11	1·5	0·09	0·9	0·08	1·0	0·5	0·02	2·9	Trace to +	—	50
	345	15	,,	0·10	1·4	0·06	0·7	0·07	0·9	0·4	0·02	3·1	+ to + +	—	60
Average	326	15	—	0·10	1·3	0·06	0·7	0·07	0·9	0·5	0·02	2·8	—	—	43
15 mg. *l*-ascorbic acid.															
	340	6	Intraperitoneal injection	0·14	1·8	0·09	1·0	0·13	1·8	0·6	0·04	6·0	+ + + to + + + +	0	40
	330	6	,,	0·12	1·6	0·11	1·1	0·09	1·4	0·6	0·03	5·4	+	0	30
Average	335	6	—	0·13	1·7	0·10	1·1	0·11	1·6	0·6	0·04	5·7	—	0	35
15 mg. *l*-ascorbic acid daily.															
	290	15	*Per os*	0·14	1·7	0·11	1·0	0·11	1·5	0·6	0·02	3·6	+ + + +	—	20
	352	15	,,	0·06	1·0	0·06	0·8	0·07	1·1	0·6	0·02	3·6	+ + +	—	90
	365	15	,,	0·10	1·6	0·08	1·1	0·07	1·1	0·5	0·02	3·5	+ to + +	—	70
	335	15	,,	0·09	1·2	0·04	0·4	0·08	0·9	0·5	0·01	2·3	+ + +	—	60
Average	336	15	—	0·10	1·4	0·07	0·8	0·08	1·2	0·6	0·02	3·3	—	—	60

Table I (*cont.*).

	Wt. f anit .l g	Scorbutic diet days	Method of administration	Quantity found: Small intestine mg./g.	Small intestine Total mg.	Large intestine mg./g.	Large intestine Total mg.	Liver mg./g.	Liver Total mg.	Adrenal mg./g.	Carcass mg./g.	Carcass Total mg.	Pituitary	Excreted in urine mg.	Gain in wt. g.
						20 mg. *l*-ascorbic acid.									
	295	6	Intramuscular injection	0·12	1·6	0·10	1·0	0·16	1·8	0·7	0·03	5·1	+ to + +	1·6	5
	305	6	,,	0·09	1·3	0·10	1·1	0·10	1·2	0·8	0·03	4·6	+ to + +	3·7	5
	275	6	,,	0·07	0·9	0·07	0·8	0·09	0·9	0·9	0·02	3·1	+ + to + + +	1·7	0
	285	6	,,	0·15	2·0	0·08	0·8	0·11	1·4	0·9	0·03	4·3	+ + + +	3·6	15
Avera	290	6	—	0·11	1·5	0·09	0·9	0·12	1·3	0·8	0·03	4·3	—	2·7	6
						20 mg. *l*-ascorbic acid daily.									
	340	15	*Per os*	0·18	2·9	0·11	1·2	0·20	3·0	1·0	0·05	8·1	+ +	—	60
	340	15	,,	0·13	2·3	0·09	1·2	0·11	1·7	0·8	0·03	4·5	+ +	—	50
	320	15	,,	0·17	2·2	0·09	0·8	0·11	1·6	0·7	0·04	6·0	+ + +	—	45
	330	15	,,	0·29	3·5	0·15	1·6	0·23	3·2	1·6	0·04	5·8	+ + + to + + + +	—	45
Avera	332	15	—	0·19	2·7	0·11	1·2	0·16	2·4	1·0	0·04	6·1	—	—	50
						25 mg. *l*-ascorbic acid.									
	320	6	Intramuscular injection	0·20	2·2	0·12	1·2	0·17	2·4	1·0	0·03	5·8	—	4·0	20
	315	6	,,	0·18	2·2	0·14	1·4	0·13	2·0	1·0	0·04	6·6	+ + + +	6·2	40
	270	6	,,	0·19	2·3	0·10	0·9	0·12	1·1	0·8	0·03	4·1	+ + to + + +	5·8	5
	277	6	,,	0·17	1·7	0·14	1·4	0·13	1·4	0·9	0·03	4·4	+ + to + + +	5·6	12
Avera	296	6	—	0·19	2·1	0·13	1·2	0·14	1·7	0·9	0·03	5·2	—	5·4	19
						50 mg. *l*-ascorbic acid.									
	290	6	Intravenous injection	0·14	1·9	0·10	1·2	0·16	2·1	0·9	0·04	5·1	+ + + +	13·0	18
						No dose.									
	[illegible]	7	—	0·04	0·6	0·02	0·3	0·07	0·9	0·1	0·00	0·0	Trace	—	10

The influence of the quantity of ascorbic acid in the tissues on its excretion in the urine.

Reference was made above to the fact that extremely high doses of ascorbic acid must be administered to guinea-pigs *per os* before it appears in the urine. This is illustrated by the following details. Guinea-pigs previously depleted of vitamin C by subsisting for 5 days on a scorbutic diet failed to excrete any ascorbic acid in the urine after receiving 9 daily doses of 50 mg. The administration of as much as 300 mg. to animals not even previously depleted failed to cause any appreciable excretion. Only when the dose was raised to 500 mg. did the vitamin appear in the urine and then in a comparatively small amount, namely 8 mg. Similarly a dose as high as 1 g. was followed by the excretion of as little as 36 mg. Euler [1935] also failed to find appreciable excretion by the guinea-pig after similar doses given *per os*.

As seen from Table I, ascorbic acid is much more readily excreted by the kidney after it is injected. It was found in fact that the vitamin began to be voided in the urine after a single injection as soon as its concentration in the tissues of the animal approached the maximum. In these experiments this was achieved when a daily dose of 20 mg. was consumed by the guinea-pigs for 15 days or after a single injection of the same dose in vitamin C-depleted animals.

It is to be noted that comparatively little ascorbic acid is excreted in the urine when 20 mg. are injected but the quantity excreted increases with the rise in the dose. The amount of the vitamin voided is, therefore, controlled not only by the concentration in the tissues but also by the size of the dose injected. If the dose is too small no significant quantities of the vitamin will appear in the urine.

Vitamin C depletion of the tissues and prolonged growth of guinea-pigs.

As a point of interest in connexion with the problem of the influence of vitamin C storage on the welfare of the guinea-pig, it is appropriate to record here the history of these animals covering the major part of their life while subsisting on vitamin C supplies which, although fully protective, do not permit of their storage in significant quantities in the tissues. A number of such guinea-pigs were examined in this connexion and a few representative cases are described here in a summarized form. All the animals to be discussed were females since the main purpose of the experiment to which they belonged necessitated the choice of this sex. The sex factor does not, however, affect the present issue. These guinea-pigs were kept on a scorbutic diet offered *ad lib.* which consisted of oats, bran, middlings, fish meal, barley meal and autoclaved full-cream milk made up from a dried powder. Besides the basal diet the animals received daily 10 ml. of decitrated lemon juice which was approximately equivalent to 5 mg. of ascorbic acid.

B 61. This guinea-pig was under observation on the above diet from 28 July 1933 to 8 November 1934. Its initial weight was 305 g. and it reached a maximum weight of 765 g. after 313 days. The animal was killed by stunning and bleeding at the end of 467 days and its tissues were examined for the presence of ascorbic acid. No significant quantities of the vitamin could be detected. Macroscopic signs of scurvy were not found at the post-mortem examination.

B 77. This guinea-pig was under observation on the above diet from 31 July 1933 to 7 July 1934. Its initial weight was 310 g. and it reached a maximum weight of 970 g. after 221 days. The animal was killed by stunning and bleeding at the end of 465 days. No significant quantities of ascorbic acid were found in its tissues and no macroscopic signs of scurvy were found at the post-mortem examination.

B 87. This guinea-pig was under observation on the above diet from 7 September 1933 to 4 May 1936, i.e. for 969 days, when it died of pneumonia. No macroscopic signs of scurvy were found at the post-mortem examination. Its initial weight was 340 g. and it reached the maximum weight of 985 g. after 649 days.

B 99. This guinea-pig was under observation on the above diet from 15 January 1934. Its initial weight was 255 g. It is still alive and growing, having reached the weight of 875 g.

The above typical experiments show that the animals could survive for long periods and reach body weights similar to those attained by fully "saturated" guinea-pigs subsisting on a diet containing cabbage *ad lib.* without having any appreciable store of vitamin C in their tissues.

The influence of vitamin C depletion in guinea-pigs on the complement of their serum.

A number of years ago it was shown by the writer [Zilva, 1919] that guinea-pigs existing on a scorbutic diet and receiving low doses of the vitamin, insufficient to protect them from scurvy but high enough to prolong the duration of the disease for several months, when immunized with a typhoid organism yielded sera with as high amboceptor and agglutinin titres as those obtained

Table II.

Diet	Wt. of guinea pig g.	Complement dilution								
		1/12	1/16	1/24	1/32	1/48	1/64	1/96	1/128	1/192
5 days on scorbutic diet	310	4	4	3½	3	2½	2	1	trace	
,, ,,	315	4	4	3½	3½	3	2	1	trace	–
,, ,,	275	4	4	3½	3½	3	2	1	trace	-
,, ,,	315	4	4	3½	3	2	1	trace	–	–
7 days on scorbutic diet	340	4	3½	3½	3	2½	2	1	trace	
,, ,,	270	4	4	3½	3	2½	2	1	trace	–
Mixed diet	450	4	4	3½	3	2	2	1	trace	
7 days on scorbutic diet	315	4	4	3½	3½	3	2	2	trace	–
Mixed diet	310	4	3½	3½	3	3	1	1	–	–
7 days on scorbutic diet	342	4	4	3½	3	2½	2	trace	trace	–
Mixed diet	320	4	4	4	3½	3	2	2	1	-
10 days on scorbutic diet	310	4	3½	3	3	2½	2	1		
Mixed diet	315	4	3½	3	3	2	1	1		
10 days on scorbutic diet	350	3½	3½	3½	3	3	2	2	1	–
Mixed diet	305	4	4	3½	3	2½	2	1½	trace	-
10 days on scorbutic diet	335	4	3½	3	2½	2	1	trace	–	–
Mixed diet	335	4	3½	3	3	2	1	trace	trace	–
19 days on scorbutic diet	380	4	4	3½	2½	2	1	trace	–	–
,, ,,	330	4	4	3½	3	2½	1	trace	–	–
Mixed diet	310	4	3½	3	2½	2	1	trace	–	–
20 days on scorbutic diet	320	3½	3	2	1	trace	–	–	–	–
,, ,,	325	4	3½	3	2	1½	1	trace	–	–
Mixed diet	305	4	3½	2	1½	1	trace	trace	–	–
7 days on scorbutic diet	440	4	3½	2	1½	1	trace	–	–	–
,, ,,	470	4	3½	2	1½	1	trace	–	–	–
Mixed diet	440	4	4	2	1½	1	1	–	–	–
7 days on scorbutic diet	425	4	3½	3	2½	2	1	trace	–	–
,, ,,	450	4	3½	3	2½	2	1	trace	–	–
Mixed diet	450	4	3½	3	2½	2	1	trace	–	–

from animals receiving cabbage *ad lib.* Nor was there any diminution in the complement activity of the blood of these animals subsisting on the restricted diet for prolonged periods. Recently Marsh [1936] asserted that in guinea-pigs depleted of vitamin C for 7 days or less the complement, as determined by a standardized haemolytic serum, either disappeared or suffered reduction. No experimental details were given.

Table II gives data of complement titrations obtained with sera from guinea-pigs which have been deprived of vitamin C for 5, 7, 10, 19 and 20 days respectively. It is seen, as would have been expected from the writer's earlier work, that no significant difference between the titres of the sera of these animals and those of control animals receiving a mixed diet with cabbage *ad lib.* could be observed.

I am indebted to Dr D. B. Steabben for checking the readings of the above titrations.

DISCUSSION.

The minimum prophylactic daily dose which is capable of protecting growing guinea-pigs of about 300 g. subsisting on a vitamin C-deficient diet from macroscopic scorbutic lesions lies in the region of 1 mg. [Zilva, 1932; Hirst & Zilva, 1933; and others] and according to Goettsch [1928] and others about double this quantity is required to obviate the development of microscopic lesions in the pulp of the teeth of these animals. The above results show that only traces of the vitamin are found in the "selective" tissues of guinea-pigs, when a daily dose of 5–8 mg.—a dose 2–3 times as great as that which prevents the production of microscopic lesions—is given for 15 days. Furthermore, to attain the maximum concentration in the tissues such as is obtained by the injection of 50 mg. of ascorbic acid in a vitamin C-depleted animal, ten times the protective dose of 2 mg. is necessary. It was found in addition that on administering the protective dose (2 mg.) to guinea-pigs on a scorbutic diet for 10 days, the time taken by these animals to succumb to the disease was not significantly less than that taken by guinea-pigs which received a daily dose of 20 mg. during the preliminary period and even by guinea-pigs which consumed cabbage *ad lib.* previous to being placed on a deficient diet. Yet in both the latter groups the concentration of ascorbic acid in the tissues was at a maximum before the vitamin was withheld from the diet. The accumulated vitamin C in the body of the guinea-pig therefore does not act as a store in the true sense of the word.

The difference between the dose necessary fully to protect the guinea-pig from scurvy and the one which brings about the maximum "saturation" of its tissues with the consequent elimination of a part of it in the urine, is therefore great. No evidence could, however, be obtained which indicated that guinea-pigs receiving daily doses ranging from the minimum protective dose to that necessary to bring about the "saturation" of their tissues with the vitamin, or even to that necessary for discrete traces of ascorbic acid to appear in the "selective" tissues, deviated from their "saturated" fellows in any discernible feature. If guinea-pigs in this zone of "unsaturation" are in a condition inimical to their well-being it is of a character too subtle to be detected by the methods employed in this investigation. Slight microscopic changes in the teeth of guinea-pigs can be observed after they have subsisted for 7–10 days on a scorbutic diet [Zilva & Wells, 1919], by which time we now know that their tissues have lost the best part of their vitamin C, but as was seen tissues of animals receiving doses capable of preventing these microscopic changes contain ascorbic acid in minimum amounts and consequently very marked depletion of guinea-pig tissues of vitamin C is no indication of the presence of even these microscopic lesions.

Great caution must be exercised in interpreting the bearing of the above results on human beings. Obviously systematic experiments such as those described cannot be carried out on man. The only information available in this respect are data recorded by a number of workers in connexion with the urinary excretion of ascorbic acid—results which are not easy to correlate. The comparison is made difficult by the fact that in determining the balance between the intake and output of the vitamin, the amount of ascorbic acid not accounted for varies with the magnitude of the ingested dose, the experimental subject and even with the same individual at different times under similar dietary conditions [cf. Johnson & Zilva, 1934]. Nevertheless, from the available evidence it may be concluded in general that in human beings suffering from declared scurvy the degree of "saturation" with vitamin C[1] is lower than that of the most "unsaturated" subjects who are free from the disease and in good and apparently normal health. This fact emerges particularly from the investigations of Schultzer [1936] and Johnson & Zilva [1934]. The observations of Archer & Graham [1936] in the writer's opinion also point in the same direction. There is so far, however, no evidence, clinical or laboratory, that such "unsaturated" subjects are in a worse state of health than "saturated" persons. The present investigation carried out on animals more susceptible to scurvy than man also failed to disclose any significant departures of the one type from the other. It was in fact found that "unsaturated" guinea-pigs receiving daily doses of vitamin C just sufficient to prevent the development of macroscopic and microscopic scorbutic lesions succumbed to the disease in almost the same time after the daily dose was discontinued as did the fully "saturated" guinea-pigs. It is also significant to note in this connexion that Schultzer's patients remained "unsaturated" for a considerable time after the disappearance of the scorbutic symptoms, in spite of receiving such high doses as 600 mg. per day. There seems therefore to be in human subjects also a wide margin of "unsaturation" which has no obvious detrimental effect on the health of the individual. To apply to this zone of "unsaturation" such expressions as "hypovitaminosis C", "latent or potential scurvy", "vitamin C sub-nutrition", "subacute scurvy" etc.—expressions which imply ill-defined pathological conditions—is not justified by our present state of knowledge. Even if some subtle inimical change be associated with this particular state of "unsaturation" the use of such expressions can only tend to obscure the issue.

It is appropriate to add that the failure on the part of an "unsaturated" subject to excrete ascorbic acid in the urine after the administration of a single dose, even a high one, does not yield any information as to the degree of his "unsaturation".

SUMMARY.

An oral dose of about 500 mg. of ascorbic acid is necessary for growing guinea-pigs (of about 300 g.) subsisting on a mixed diet before the vitamin is excreted in the urine. In contrast to this, excretion is brought about by the injection of a very much lower dose.

Graded doses of ascorbic acid were injected into vitamin C-depleted guinea-pigs and it was found that demonstrable traces of the vitamin appeared in the

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[1] The expression "saturation" is used in the sense defined by Johnson & Zilva, i.e. an animal is said to be "saturated" when upon ingesting a constant daily amount of ascorbic acid it excretes a more or less constant daily amount of the vitamin in the urine.

tissues only when a dose between 5 and 8 mg. was employed. The injection of 20 mg. of the acid produced a concentration in the tissues equal to that observed when 50 mg. were injected and this was considered the maximum concentration attainable under the experimental conditions described. Similar results were obtained when corresponding doses were administered *per os* for 15 days to guinea-pigs on a scorbutic diet. Ascorbic acid first appeared in the urine when 20 mg. were injected, i.e. when the maximum concentration of the vitamin in the tissues was reached. As the dose was increased the amount of ascorbic acid excreted also rose.

The time taken by guinea-pigs to succumb on a scorbutic diet after having previously received for 15 days whilst on this diet 2 mg. of ascorbic acid (the protective dose) daily was about the same as that taken by animals which were offered cabbage *ad lib.* during the preliminary period. Previous "saturation" of the tissues therefore does not delay the fatal termination of the disease.

Guinea-pigs on a scorbutic diet receiving daily doses of 10 ml. of decitrated lemon juice (about 5 mg. of ascorbic acid) were observed to live for a number of years during which time they attained very high weights, although the tissues of such animals contained only minimum amounts of vitamin C.

The complement activity of guinea-pig blood suffers no reduction when the animals are depleted of vitamin C or even when they are in a scorbutic condition.

Thanks are due to Messrs Hoffmann La Roche, Ltd., for a gift of ascorbic acid.

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CCIII. CATARACT AND ASCORBIC ACID IN THE GUINEA-PIG EYE.

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The occurrence of vitamin C in the aqueous humour and lens in relatively large amounts, first noted by Birch & Dann [1933; 1934] in the case of the ox and sheep, has attracted much attention by workers interested in the genesis of cataract. Thus Müller *et al.* [1933; 1934; 1935] found that the vitamin content of both the aqueous humour and lens of cattle and rabbits decreased with age and on the development of cataract. The latter workers as well as Fischer [1934] not only associated the vitamin with the respiratory activity of the lens but suggested that it was most probably synthesized in that organ, at least of young animals. However, although Fischer found that derangements of the respiratory function resulted in the formation of cataracts, he was unable to state whether the disappearance of the vitamin preceded or followed the development of the cataract. The view that the lens could synthesize the vitamin was questioned by Van Eekelen *et al.* [1934] who inclined to the belief that it was accumulated there by a selective secretory process.

Similar variations in the vitamin content of the eyes of other animals and man due to the same causes have been observed by Euler and co-workers [1933; 1934].

The above results were obtained chiefly by titration of extracts with indophenol but the presence of the vitamin in the humours and lens of the normal eyes of the ox was demonstrated biologically by Birch & Dann [1934], Euler & Malmberg [1934] and more recently by Müller & Demole [1935].

It is now well established that the guinea-pig quickly loses its store of vitamin C when maintained on a scorbutic diet. The organs of this animal which are known selectively to absorb it have been found to lose their individual stores simultaneously and to become depleted in about the same time. Since the amount of vitamin available for distribution in the body of the guinea-pig can be readily controlled, it was of interest to determine whether the eye behaved similarly to the other organs in this respect in the hope of contributing to the solution of the above problem.

The problem was considered from two aspects. One the fate of the ascorbic acid in the eye of the guinea-pig during the process of depletion of vitamin C and the other its occurrence in the eyes of the depleted animals following the administration of the vitamin.

Owing to the small quantities of material available, biological methods of establishing the presence or of determining the amounts of vitamin C in the lens of the guinea-pig were not applicable to the present work. The indophenol titration method whilst simple tends to give untrustworthy results when used with extracts of tissues, such as the lens, which contain sulphydryl compounds. The spectrophotometric method was therefore employed in conjunction with the titrimetric method in order to obtain greater specificity.

Trichloroacetic acid, which was used by Van Eekelen *et al.* [1934] and Plaut *et al.* [1935] as a protein-precipitating agent in their work on the humours and cerebrospinal fluid, is not suitable for the preparation of extracts in these determinations because of the opacity of its solutions in the region below about 260 $m\mu$ where ascorbic acid shows selective absorption. Freshly redistilled absolute alcohol was therefore employed not only on account of its transparency but also because of the stability which it confers upon ascorbic acid.

EXPERIMENTAL.

The spectrograph, light source etc., which were employed in this work have already been described in another paper from this laboratory [Kellie & Zilva, 1936].

A value of 10,000 was obtained for the molecular extinction coefficient of pure ascorbic acid in $N/100$ HCl in 90 % alcohol. In this solution the head of the absorption band occurred at 245 $m\mu$. All determinations were made on acidified solutions and the above value was used in estimating the amounts of ascorbic acid.

Titrimetric and spectrophotometric determinations of ascorbic acid.

The humours and lenses of the horse, ox, sheep and pig, as well as those of the guinea-pig, were examined.

Determinations in the aqueous humour. The humours were collected in an injection syringe by puncture of the cornea near to the centre of the pupil, the needle being inserted obliquely so as to avoid injury to the iris and lens. They seldom needed to be clarified but when necessary they were filtered or centrifuged.

For the titrimetric determination the humour was acidified with one-fifth of its volume of glacial acetic acid and titrated directly with indophenol. The latter was standardized so that 10 ml. of the indicator solution were equivalent to 1·0 mg. of ascorbic acid.

In the spectrographic determination a dilution of 1 in 10 generally sufficed to give the required concentration of 0·5–2·5 mg. of ascorbic acid per 100 ml. Two equal volumes of humour, usually 1·0 ml., were necessary. One was immediately acidified with 0·2 N HCl and diluted with fresh glass-distilled water to give the desired concentration of humour in 0·02 N HCl. The ascorbic acid in the second sample was oxidized by diluting it with water to about 80 % of its final volume and adding sufficient $CuSO_4$ solution to give ultimately a concentration of 2 mg. of Cu per litre of solution. At the reaction of these solutions, which was invariably neutral, the oxidation was complete in an hour. It was then acidified and the acid concentration adjusted as in the previous case. The absorption spectrum of each solution was measured by comparing the first with an equal depth of 0·02 N HCl and the second with a similar solution containing 2 mg. of Cu per litre. This procedure allows the absorption due to the reagents to be balanced during the determinations and thus to be virtually eliminated from the photographs. The difference between the curves for the two solutions gives a measure of the absorption due to ascorbic acid.

Determinations in the vitreous humour. The vitreous humour and lens were removed together by pressing in the cornea after slitting the sclerotic. They could then be easily separated from one another. The humour was freed from the fine fibrous mesh and other insoluble material by filtering it with gentle suction through a small tight pad of glass wool. Any excess protein in the filtrate could be readily precipitated by adding one drop of glacial acetic acid per ml. of humour.

The spectrophotometric and titrimetric determinations were carried out in the same manner as in the case of the aqueous humour.

Determinations in the lens. The lenses were freed from adhering humour with filter-paper. Titrations were carried out on trichloroacetic acid extracts, which were prepared by thoroughly grinding the lenses in the first place with powdered glass and an equal weight of water and then with five volumes of 5 % trichloroacetic acid.

Alcoholic extracts were used for the spectrophotometric determinations and in calculations the lenses were considered to contain 60 % of water. The lenses were ground with powdered glass and 40 % of their weight of water. The well ground mass, whilst vigorously stirred, was slowly diluted with freshly redistilled absolute alcohol until the concentration of the latter became 90 %. The protein, which commenced to separate when the concentration of alcohol rose above 50 %, was removed on the centrifuge. Other material which reduced the transparency of the solutions in the essential ultra-violet region was precipitated by the addition of 2 % of a 1·5 % solution of $CdCl_2$ in 98 % alcohol. The final extracts were invariably neutral in reaction.

Observations made at the essential stages indicated that no appreciable losses of ascorbic acid occurred during the extraction. Table I shows that ascorbic acid in a solution of the lens material was quite stable and almost completely protected from oxidation by relatively large amounts of Cu at *p*H 7·6. There was therefore little risk of it becoming oxidized whilst the lenses were being ground. The coincidence of the absorption curves in Fig. 1 demonstrates that no significant losses occurred during the subsequent manipulations. One of the curves refers to an extract of the lens to which ascorbic acid was added before precipitation with alcohol and the other to an extract of the same material to which an equal quantity of ascorbic acid was added after the precipitation. Such agreement between the two curves is only possible when no loss of ascorbic acid is involved in the precipitation.

Table I.

Time in hours	ml. *N*/1000 indophenol reduced by 1·0 ml. solution containing 13 mg. ascorbic acid per 10 ml. of 2 % lens material Solution kept at *p*H 7·6: Solution alone	Solution containing 0·1 mg. Cu per 10 ml.
0	15·5	15·5
3	14·0	14·0
7	13·0	13·0
20	12·0	10·0

In the spectrographic determination, the oxidation was carried out by adding sufficient Cu to give a concentration of 4 mg. per litre. Although ascorbic acid is normally quite stable in alcoholic solution it oxidizes in alcohol at a much quicker rate than in water on the addition of Cu, presumably owing to the much larger amounts of oxygen dissolved by the former. The freshly prepared and oxidized extracts were acidified with 25 % of their volume of 0·2*N* HCl. When each extract was balanced against its corresponding blank solution, as in the case of the humours, more satisfactory results were obtained with the extracts of the lens of the horse, ox, sheep and pig than with those of the guinea-pig. In contrast to the others it was found that in the guinea-pig extracts the absorption due to ascorbic acid was small compared with the total absorption. This difficulty was overcome by comparing the oxidized and untreated extracts together in the photometer, which virtually eliminates the absorption due to the material other than ascorbic acid in the extracts. It was then necessary to reduce the amount of Cu to 1/10th of that previously employed and to determine its absorption also, for although the latter is very small in dilutions of this magnitude it cannot be ignored when the absorption to be measured is not great.

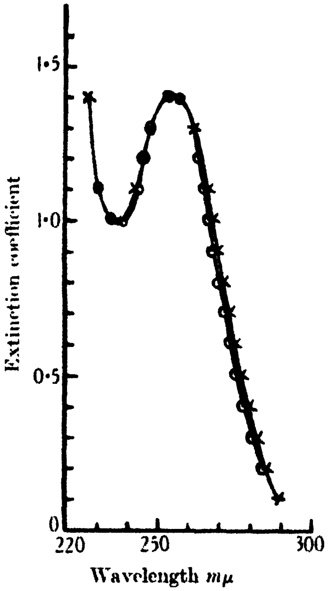

Fig. 1.

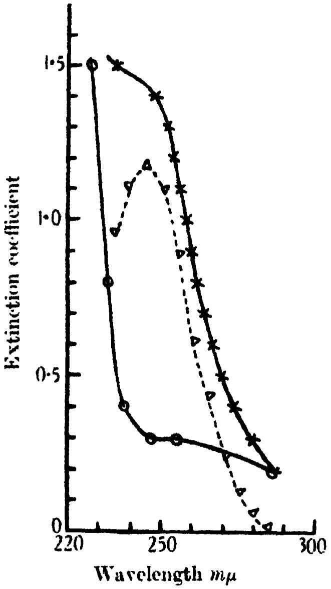

Fig. 2.

Fig. 1. Guinea-pig lens extract containing 12·5 mg. added ascorbic acid per 100 g. of lens. Extract equivalent to 4 g. lens per 100 ml. ×—× Ascorbic acid added before precipitation. o—o Ascorbic acid added after precipitation.

Fig. 2. Ox aqueous humour, dilution 1 10. Ascorbic acid = 21 mg. 100 ml. (titration showed 21·6 mg./100 ml.). ×—× Humour. o—o Humour after oxidation. △—△ Ascorbic acid.

RESULTS.

Good agreement between the results of the spectrophotometric and indophenol titration methods was found to exist only in the case of the humours. The titration values obtained for the lenses were invariably higher than those of the spectrographic method (Table II). The absorption curves in Figs. 2, 3 and 4 are typical and illustrate this fact. In this connexion it must be mentioned that the biological tests of Müller & Demole [1935] showed that whereas the ascorbic acid content of the humours of the ox eye was truly indicated by the indophenol method, that of the lens only accounted for about a half of its indophenol-reducing capacity. It may therefore be concluded that whilst the titrimetric method could not be applied in the determination of ascorbic acid in the lens, the

Table II.

Animal	Tissue	Ascorbic acid in mg. per 100 ml. or per 100 g.: Indophenol titration	Ascorbic acid in mg. per 100 ml. or per 100 g.: Spectrophotometric determination
Horse	Aqueous humour	16·6	18·0
	Vitreous humour	9·0	12·0
Ox	Aqueous humour	21·6	21·0
	Vitreous humour	15·0	10·0
Sheep	,, ,,	13·5	10·0
Pig	,, ,,	5·0	5·5
Guinea-pig	,, ,,	21·3	20·0
,,	,, ,,	18·5	19·0
Horse	Lens	37·0	26·0
Ox	,,	32·6	22·5
Sheep	,,	21·0	17·5
Pig	,,	26·0	13·5

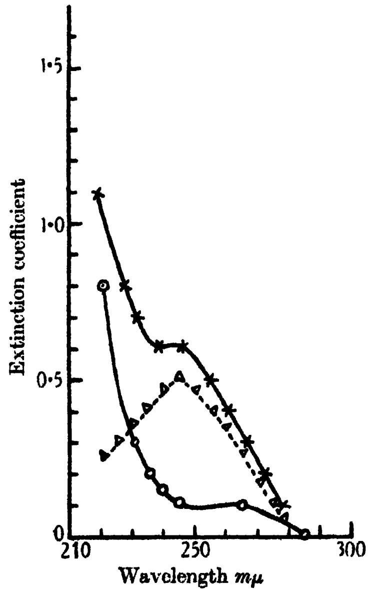

Fig. 3.

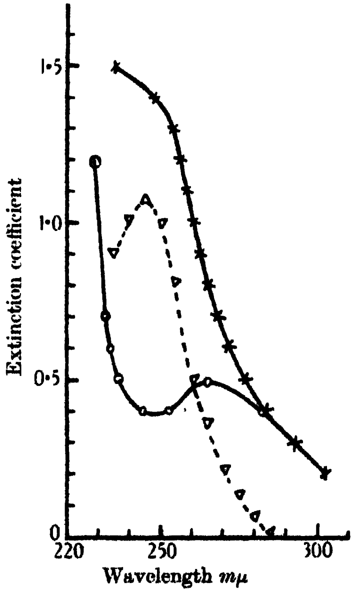

Fig. 4.

Fig. 3. Ox lens extract, equivalent to 4·4 g. lens per 100 ml. Ascorbic acid = 22·5 mg./100 g. lens (titration showed 32·6 mg./100 g. lens). ×—× Extract. o—o Extract after oxidation. △—△ Ascorbic acid.

Fig. 4. Guinea-pig vitreous humour, dilution 1/10. Ascorbic acid = 19 mg./100 ml. (titration showed 18·5 mg./100 ml.). ×—× Humour. o—o Humour after oxidation. △—△ Ascorbic acid.

spectrophotometric method showed adequate specificity for this purpose. Consequently the titration method sufficed in the case of the humours but both methods were employed for comparison in the examination of the lens.

The fate of ascorbic acid in the humours and lens of the guinea-pig during depletion of vitamin C.

The guinea-pigs employed in this work were taken from a stock which had subsisted for a long period on a normal mixed diet of oats, bran and cabbage *ad lib.* They were divided into three groups each consisting of 12 animals of similar age and weight. The eyes of the first group were used as controls and were examined immediately. The second and third groups were maintained on scorbutic diets for 5 and 9 days respectively. All the animals were killed by stunning and bleeding and their eyes removed and examined without delay. No macroscopic abnormalities were observed in any of the eyes.

Table III.

Group	Average weight g.	Diet	Duration of experiment days	Titrimetric determination ascorbic acid in humours mg. per 100 ml.		Ascorbic acid in lens mg. per 100 g.	
				Aqueous	Vitreous	Titrimetric determination	Spectrophotometric determination
1	390	Normal	—	13·0	12·0	8·0	4·0
2	398	Scorbutic	5	0·5	2·5	4·7	1·5
3	404	"	9	0·5 (?)	0·7	4·0	0·0

It will be seen from Table III that the concentrations of ascorbic acid in the two humours of the animals of the control group were about equal but greater than that in the lens. In the latter the concentration of the vitamin determined spectrographically amounted to only half of that indicated by the titration. In group 2 the concentration in the lens as determined by both methods had fallen but the titration value remained much higher than that of the spectrographic method. The value found by the spectrograph in this case is only approximate owing to the low level to which the concentration had fallen. The loss of the vitamin from the humours of the animals of this group was also very marked: it was almost complete in the aqueous humour but an appreciable amount remained in the vitreous humour. No ascorbic acid was detectable by the spectrographic method in the lenses of the last group of animals in spite of the fact that the indophenol titration method indicated a reducing capacity equal to half that of the control group. This residual reducing capacity happens to be equal to the difference between the amounts of ascorbic acid determined by titration and by the spectrograph in the case of the animals of the control group which strongly indicates that the fall in the reducing capacity during the period of depletion is due to ascorbic acid alone.[1] Both the humours of the last group were practically devoid of ascorbic acid.

The above results show that the humours and lens resemble the other tissues in their inability to conserve their stores of ascorbic acid when the animals are maintained on vitamin C-deficient diets. It is concluded from the rate at which it occurs that the loss of the vitamin from the eye runs parallel with that of the body generally. This conclusion is contrary to the view expressed by Ray *et al.* [1935] that the lens of the scorbutic guinea-pig holds tenaciously to a part of its vitamin C content. They arrived at this conclusion by the application of the titrimetric method only. In view of the above results the residual indophenol-reducing capacity of the lens observed by them, which in any case seems rather high, was evidently not due to ascorbic acid.

The occurrence of ascorbic acid in the eyes of vitamin C-depleted guinea-pigs following its administration.

The distribution of the vitamin in the avascular parts of the eye can be approximately followed from the ascorbic acid concentrations in the humours which nourish them since it has been shown that the lens and humours are analogous in their behaviour to one another and to the other tissues which selectively absorb it. In order therefore to simplify the experimental procedure, titrations of the vitreous humour only were carried out in the following experiments. Seven groups of two animals each, similar to those previously employed were used in this work. They were all maintained on a scorbutic basal diet. After the preliminary period of depletion on this diet the animals of three of the groups received ascorbic acid *per os* and the remainder by intramuscular injection. In all cases the animals were killed in the same manner as those in the previous experiment 24 hours after being given their last dose. The eyes of all the animals, none of which showed any clinical signs of scurvy, appeared to be quite normal at the end of the experimental periods. I am indebted to Dr S. S. Zilva for having kindly placed at my disposal for this work the eyes of animals which were being used by him in another investigation.

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[1] Only approximate relations between the corresponding figures can be expected since the changes occurring in the eyes cannot be examined in the same animals at each stage.

Table IV.

Group	Average weight g.		Preliminary period on scorbutic diet days	Daily dose of ascorbic acid mg.	Number of doses	Method of administration	Ascorbic acid in vitreous humour mg. per 100 ml.
	Beginning of exp.	End of exp.					
1	283	396	10	0·5	20	*Per os*	0·5
2	270	365	6	15·0	14		16·0
3	293	320	5	25·0	3		8·0
4	265	298	5	8·0	1	Intramuscular injection	5·0
5	275	290	5	5·0	1		3·0
6	263	279	5	8·0	1		7·0
7	266	280	5	5·0	1		4·0

Table IV shows that in the animals in group 1, which were given a prophylactic dose of 0·5 mg. of ascorbic acid for 20 days after a preliminary period of depletion lasting for 10 days, only minimum amounts of ascorbic acid had been accumulated in the vitreous humour. The concentration of the vitamin in the humour of the second group of animals was about normal at the end of 14 days. They had received thirty times the prophylactic dose daily after a much shorter period of depletion. The animals of the third group were depleted for 5 days and then given 25 mg. of the vitamin on each of the following 3 days. In their case the concentration in the humours rose to only half of that of the previous group. The remaining animals, which received a single dose by injection on the 6th day, were arranged in two pairs of groups so that the second pair were duplicates of the first. One group in each pair was given 8 mg. and the other 5 mg. As will be seen from Table IV, the concentration in the humour appeared to rise more rapidly on injection than after oral administration. These latter results are in consonance with those found by Zilva [1936] for other tissues of the same animals.

The manner in which the concentration of ascorbic acid in the vitreous humour of vitamin C-depleted animals varies with the method, size and frequency of administration of the dose is strictly comparable with that which has been observed to occur in other organs and tissues under the same conditions.

The loss of indophenol-reducing capacity and characteristic absorption in the lens and humours is unlikely to be due to the conversion of the vitamin into dehydroascorbic acid for the following reasons. It was seen that the lens material, like many other tissues, exercised a marked stabilizing action on ascorbic acid in solution, protecting it from oxidation. Further, Goldmann & Buschke [1935] state that dehydroascorbic acid is reduced by the lens when it is introduced into the aqueous humour. None of the tissues so far examined has been found capable of dehydrogenating ascorbic acid *in vitro* [Kellie & Zilva, 1935]. Finally, when dehydroascorbic acid is administered the body was shown to reduce it [Johnson & Zilva, 1934].

SUMMARY.

Good agreement was found between the indophenol titration and spectrophotometric methods of determining the concentration of ascorbic acid in the humours of the eyes of the horse, ox, sheep, pig and guinea-pig. In the case of the lenses, however, indophenol titration invariably gave results which were higher than those of the spectrographic method.

In guinea-pigs on scorbutic diets, the indophenol-reducing capacity of the humours fell rapidly and was almost nil after 9 days. At the end of the same period there remained in the lens some indophenol-reducing capacity which was shown by the spectrograph not to be due to ascorbic acid.

The level of concentration of ascorbic acid in the humours of vitamin C-depleted animals can be raised by the injection or oral administration of ascorbic acid.

The rates of disappearance of the vitamin from the lens and humours during depletion and of its reappearance in the humours on readministration run parallel therefore with those of the other tissues of the guinea-pig.

No cataracts were observed in any of the eyes of the apparently normal animals (those which were maintained for short periods on scorbutic diets) whose humours and lenses were found to be devoid of vitamin C, or even as late as the premortal stage in groups of guinea-pigs suffering from well declared scurvy.

The improbability of the presence of dehydroascorbic acid in the lens and humours is stressed.

It is concluded that the deprivation of guinea-pigs of vitamin C has no direct bearing on the aetiology of cataract in these animals.

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CCIV. STUDIES ON YEAST GROWN IN CYANIDE. II.

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In the previous communication [Pett, 1935, 3] the observation was reported that yeast could be grown in media containing cyanide. The resulting yeast was found to have a high fermentative capacity, and a respiration largely insensitive to cyanide. It contained several times the normal content of flavin, in the form of flavoprotein.

The present communication gives a comparison of cyanide-yeast with yeast experimentally grown aerobically (like baker's yeast), and anaerobically (like bottom yeast), together with observations on the effect of prolonged subculture in cyanide. The results show definite differences in the three classes of yeast; prolonged subculturing in cyanide resulted in the disappearance of flavin from extracts of the yeast, with the appearance of a blue fluorescence. Some of the properties of this yeast are given.

EXPERIMENTAL.

Medium. This contained, in g. per litre, KH_2PO_4 1; $MgSO_4, 7H_2O$ 0·5; NaCl 1; $(NH_4)_2HPO_4$ 4; marmite 0·5; glucose 20. The *p*H was adjusted to 5 before sterilizing and determined after growth, being usually 3·0–3·5.

Apparatus. 500 ml. of medium were used in a 1 litre Erlenmeyer flask, vigorously aerated through a Jena sintered glass device, or by one constructed from a Gooch crucible. The air was filtered through cotton wool in an S-shaped tube of 1 cm. bore and 50–60 cm. length. The stopper sometimes carried a separating funnel for removing samples at intervals. The stopper, complete, was wrapped and autoclaved separately, being aseptically inserted after inoculation of the flask, in a special room. The first absorption tube contained 40 % NaOH, the subsequent ones *N* NaOH. The incoming gas, either air or nitrogen largely freed of oxygen by pyrogallol and of CO by a copper solution, was drawn through a gas wash-bottle containing either water or slightly acidified *M*/750 NaCN solution replaced every 12 hours. This procedure maintained the medium at *M*/1000 to *M*/1400 NaCN. Concentrations higher or lower can be produced by suitable variations of the solution, and frequent changes of the solution permit control of the cyanide in the medium within fairly narrow limits.

Under our conditions suction maintained a steadier flow of gas than pressure and was always used. All connexions were made with pressure tubing. The whole apparatus stood in an incubator at 26°, and the yeast was grown for 48 hours unless specified. Erlenmeyer flasks are not ideal for good aeration but were the most suitable of the standard glassware in possessing a large neck to admit the apparatus, and surplus room for frothing.

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[1] This work was carried out during the tenure of an Overseas Scholarship from the Royal Commission for the Exhibition of 1851. The author is now in the Department of Biochemistry, University of Alberta, Edmonton, Canada.

Procedures. Cyanide determinations were made at frequent intervals on the various solutions of the system using the sodium mercuric chloride titration to methyl yellow of Kolthoff & Furman [1929].

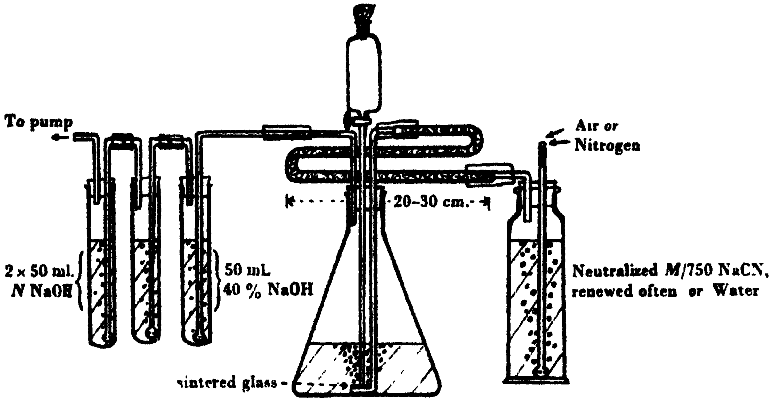

Fig. 1.

Respiration and fermentation were determined in the Warburg micromanometric apparatus and are expressed as Q_{O_2} and Q_{CO_2} (µl. gas change in 1 hour for 1 mg. dry weight of yeast). The bath was set at 30°, which is the upper limit of the optimum temperature range for growing yeast. The general reproducibility of the results at different times, using different lots of yeast etc., was never better than 5 %. The yeast was suspended in 0·05 M KH_2PO_4. Fresh, neutralized KCN solution was used to give an ultimate concentration of $M/600$. The ultimate concentrations of the various substrates used were: glucose 2 %: lactate 1·66 %: alcohol 1·66 %. Nitrogen was freed from oxygen by passing over glowing copper. Fluted filter-paper (Whatman No. 40) was used in the alkali, and when cyanide was used the alkali also contained cyanide [see Van Heyningen, 1935]. This precaution is actually not important with yeast at 30° for a short time, since repeated parallel experiments without cyanide in the alkali still gave the same Q_{O_2}. The pH of the mixture in each vessel was checked after the experiment.

Flavin was determined in a concentrated beam of light as described by Pett [1935, 3].

RESULTS.

Preparation of the yeast. The effect of aeration.

The yeast was centrifuged and washed 5 or 6 times with distilled water. The custom of impoverishing the yeast by vigorous aeration for 4–5 hours in buffer at pH 7, followed by additional washings, was sometimes followed. This procedure, however, is open to serious criticism. The metabolic capabilities of starved yeast are not the same as of the original yeast in several respects, though it has been assumed that the only change is a depletion of the glycogen reserves. This leads to a low Q_{O_2} without added substrate and makes various experimental effects more striking. The fact is that other changes occur. Of importance to the present discussion is the disappearance of flavin during the treatment as shown in Table I, taken from Pett [1935, 2]. Even more rapid disappearance is often observed.

Table I. *Changes in flavin content of baker's yeast during aeration in buffer.*

Time in hours	0	4	8	12	24	36
Flavin γ/ g.	24	16	12	10	7	6

Clearly glycogen is not the only constituent affected by aerating the yeast, and the subsequent reactions of the yeast may thus not be "normal". This idea receives further support from observations on the respiration of yeast before and after aeration. Table II shows typical results of the effect of cyanide on the respiration before and after aeration of some commercial baker's yeast and on some similar yeast experimentally grown under aerobic conditions.

Table II. *The increased sensitivity to cyanide of the respiration of baker's yeast and similar experimentally-grown yeast, after aeration in buffer.*

	Baker's yeast		Experimental yeast	
	Before aeration	After aeration	Before aeration	After aeration
No substrate Q_{O_2}	− 6	− 2	−31	− 4
$Q_{O_2}^{CN}$	− 6	− 3	−27	− 5
Glucose Q_{O_2}	−92	−89	−32	−25
$Q_{O_2}^{CN}$	−21	−11	−19	− 5

Before aeration, especially without substrate, when the yeast is using its natural reserves, the respiration is less sensitive to cyanide. This is clearer in the fresher, experimentally grown yeast. Ogston & Green [1935] have also pointed out the CN-stability of the low natural respiration of yeast. Percentage inhibitions are not given because they give various results depending on the method of calculation. Methods in the literature could show either 40 or 100% inhibition in Table II.

Effect of the growing time on respiration, fermentation, glutathione and flavin of various yeasts.

Large flasks were used (see Fig. 1) and samples were aseptically removed from the flasks at stated intervals and examined. The results are given in Table III.

Table III. *The effect of the growing time on the respiration, fermentation, glutathione and flavin content of yeasts grown aerobically, anaerobically and aerobically with cyanide.*

		2 days			3 days			5 days		
		Aerob.	Anaerob.	CN	Aerob.	Anaerob.	CN	Aerob.	Anaerob.	CN
No substrate	Q_{O_2}	− 6	− 5	− 2	− 5	−15	− 2	− 2	−11	− 2
Glucose	Q_{O_2}	−44	− 41	−10	−24	—	−15	−13	−17	−15
	$Q_{O_2}^{CN}$	− 3	− 18	− 9	− 6	− 7	−11	− 2	− 5	−11
Lactate	Q_{O_2}	−26	− 22	− 5	−37	− 5	− 2	−25	−14	—
	$Q_{O_2}^{CN}$	−22	− 2	− 9	−14	− 2	− 6	−20	− 2	—
Alcohol	Q_{O_2}	−38	− 24	− 7	−18	− 8	− 2	−47	− 8	—
	$Q_{O_2}^{CN}$	− 3	− 2	− 2	− 2	− 1	− 2	−22	− 1	—
Glucose	Q_{CO_2}	+25	+120	+76	+18	+35	+39	+16	+52	+15
	$Q_{CO_2}^{N_2}$	+47	+148	+78	+37	+42	+30	+24	+58	+10
Flavin γ/g.		17	13	51	12	12	48	15	15	50
Glutathione mg./100 g.	SH	—	—	—	120	47	46	170	151	115
	Total	—	—	—	121	49	153	175	156	250

In glucose the highest respiration and fermentation were found in the yeast grown the shortest time (2 days). With lactate or alcohol as substrate no general trend was noticed. Similarly the relative flavin concentrations did not change significantly, though a change in the glutathione (only twice determined) was found.

The Meyerhof quotient has been calculated but varied in a very irregular manner depending on purely fortuitous variations in the figures. Its calculation implies in such figures an absolute accuracy and significance which they do not possess.

General properties of cyanide-yeast.

The medium was always inoculated with 1 ml. of a 2-day culture of *S. cerevisiae*, grown on the same medium. Several different strains have been successfully tried, but only one was used throughout the present work. To produce cyanide-yeast, HCN-laden air was passed through the system previously described. Aseptically adding cyanide to the medium was difficult and unnecessary since the medium took up cyanide from the air in a few hours. Three controls were grown simultaneously: (1) aerobic, by passing ordinary air, (2) anaerobic, by passing nitrogen, (3) anaerobic-cyanide (called N.C.N.) by passing nitrogen through cyanide solution. It is desirable to pass gas through the flasks in this way in order to remove CO_2 and to keep the medium in motion, thus making a truer comparison with the aerobic and aerobic-cyanide yeasts. The *p*H at the end was never less than 3·0 unless bacterial contamination had occurred. Microscopical examination both by staining and on a dark ground was a routine: only rarely were bacteria found, in which case the duplicate flask only was used.

The properties of yeasts cultured in this way may be seen in Tables III and IV (1st series). A high content of flavin must be present for typical CN-yeast as here discussed, though subculturing in cyanide may produce a flavin-free yeast which might also be called a cyanide-yeast. If a high flavin content was not found the observed properties were like those of aerobic yeast. The Q_{O_2} in glucose and lactate was about 1/4 that of aerobic yeast and was scarcely affected by cyanide. The Q_{O_2} in alcohol was also small, but might show marked CN-sensitivity. The fermentation was usually higher than that of aerobic yeast, however determined. Cyanide-yeast has a high aerobic fermentation and this is not increased by determination in nitrogen. In other words the usual increase of anaerobic fermentation over aerobic (the Pasteur effect or reaction), which is observed in the control yeasts, was not found in CN-yeast.

The Q_{O_2} of all these yeasts may appear small to those accustomed only to commercial yeast, but is really quite high for experimentally grown yeast, especially on synthetic medium.

The glutathione content of these yeasts was twice determined but the results are only tentative. CN-yeast contained significantly more glutathione than the controls, but whereas the controls had it all in the —SH form, the CN-yeast had only about 1/3 as —SH. The high proportion of the oxidized form is the more remarkable since it has been suggested that oxidation of glutathione in animal tissues requires a CN-sensitive iron catalyst, and also since *in vitro* cyanide reduces GSSG. Possibly some other substance was produced and was determined by the unspecific method used.

Unfortunately quantitative measurements of cytochrome have not yet been possible, owing to the small amount of yeast available and the need for about 1 g. to make the estimation. The yield from 500 ml. medium has been from 0·5 to 1·5 g. fresh yeast, which was almost all required for the duplicate determinations of flavin, respiration etc. The question is therefore open whether cytochrome still functions in CN-yeast by making use of some CN-insensitive enzyme, but it is generally held that cytochrome is exclusively and specifically oxidized by a CN-sensitive enzyme.

The fate of the cyanide is still largely unexplored, but in all cases 1/3 or more of the amount introduced disappears from the flask containing yeast, though this does not represent more than perhaps 50 mg. NaCN. Evidence of disappearance of cyanide in Warburg vessels has also been observed, often with increased O_2 uptake over controls. This suggests production of cyanates. It is possible that other reactions take place, such as formation of cyanohydrins. In this case further reaction must take place since no increased glycerol production was found, as would be expected from the familiar "trapping methods" used in yeast fermentation studies.

The effect of subculturing in cyanide.

Marked changes have been found as a result of this treatment. A series of 4 flasks, in which yeast was to be grown aerobically, anaerobically, anaerobically with cyanide and aerobically with cyanide, called the 1st cyanide series, was inoculated from the 2-day cyanide flask of an ordinary series. After 2 days' growth a new series, called the 2nd cyanide series, was inoculated from the CN-yeast of the 1st series. The 1st series was then centrifuged, washed and examined as usual. After 2 days a 3rd cyanide series was inoculated with the CN-yeast of the 2nd, etc.

The questions involved are whether yeast once grown as CN-yeast reverts on subsequent culturing aerobically or anaerobically, and whether culturing in

Table IV. *Effect of subculturing on certain properties of yeasts inoculated with cyanide-yeast, then grown aerobically, anaerobically, anaerobically with cyanide and again aerobically with cyanide.*

The 1st series was inoculated from the cyanide-yeast of an ordinary series, the 2nd series from the CN-yeast of the 1st, the 3rd series from the 2nd, etc.

		No substrate		Glucose		Lactate		Alcohol		Glucose		Flavin
Series		Q_{O_2}	$Q_{O_2}^{CN}$	Q_{O_2}	$Q_{O_2}^{CN}$	Q_{O_2}	$Q_{O_2}^{CN}$	Q_{O_2}	$Q_{O_2}^{CN}$	Q_{CO_2}	$Q_{CO_2}^{N_2}$	γ/g.
1st	Aerobic	- 4	- 5	-14	- 1	- 6	- 3	-13	- 1	+ 82	+104	39
	Anaerobic	- 7	-10	- 8	- 5	- 9	- 4	- 5	- 5	+130	+118	36
	N.C.N.	- 4	- 4	-12	- 1	-13	- 1	-11	- 1	+ 95	+104	40
	CN	-12	-10	-12	-11	- 3	- 3	- 6	- 6	+ 81	+ 76	60
3rd	Aerobic	- 3	- 4	-18	- 7	- 7	- 1	-14	- 4	+ 31	+ 51	40
	Anaerobic	-11	- 6	- 7	- 7	-12	- 6	-10	- 6	+104	+ 46	39
	N.C.N.	- 7	- 8	-19	-14	-12	-15	-16	- 3	+214	+120	32
	CN	-49	-40	-21	-21	-48	-21	-10	- 5	+170	+152	52
5th	Aerobic	-21	-25	-42	-31	—	—	—	—	+ 73	+ 90	11
	Anaerobic	-17	-11	-10	- 2	—	—	—	—	+119	+120	12
	N.C.N.	-21	-17	-25	-23	—	—	—	—	+112	+ 93	24
	CN	-23	-24	-21	-23	—	—	—	—	+ 43	+ 43	49
15th	Aerobic	-16	-23	-51	-57	-19	-19	-48	-16	+ 54	+106	0
	Anaerobic	-27	-11	-24	-16	- 9	-10	-25	-12	+124	+102	0
	N.C.N.	-20	-12	-26	-14	-24	-11	-32	- 8	+174	+172	0
	CN	-11	-14	-34	-54	-33	-31	-53	-25	+ 93	+ 72	0
20th	Aerobic	-18	-20	-37	-42	-20	-24	-40	-22	+ 57	+ 86	0
	Anaerobic	- 8	- 4	- 9	- 4	- 9	- 4	-12	- 4	+136	+122	0
	N.C.N.	- 7	- 4	- 9	- 7	- 9	- 6	-10	- 6	+118	+166	0
	CN	-12	-18	-25	-34	-11	-16	-29	-14	+134	+165	0

cyanide would cause changes. The results of some of these series are shown in Table IV. The intervening series (not shown) carry out the indicated trends in a regular manner.

Several things are to be noticed in Table IV. The flavin, which was high in the inoculum, was slightly lower in the aerobic, anaerobic and N.C.N.-yeasts, but still high in the CN-yeast. This suggests a disturbed mechanism not yet returned to normal. At about the 5th series the flavin was decreasing, and the same alcohol extract began to show a blue fluorescence. Estimation of the flavin green was made through a blue filter. The flavin had disappeared from all yeasts by the 10th series, and a strong blue fluorescence was found. In view of the apparent ability of some bacteria to transform flavin into a blue-fluorescing substance [Pett, 1935, 1, 3] it seemed possible that contamination had occurred. Owing to the complicated handling involved this might easily have happened, but careful microscopic examination of every flask revealed no bacteria, until the 23rd series (not shown).

The Pasteur effect continued to be absent from CN-yeast but reappeared immediately (1st series) in weakened form in the aerobic yeast, but not in the anaerobic, which shows only a slight effect in ordinary conditions. Fermentation in nitrogen was less than in air (3rd and 5th series).

In all cases respiration without substrate was not greatly inhibited by $M/600$ cyanide and in most cases was not increased with substrate. Addition of cyanide with substrate was sometimes inhibitory, though the opposite, namely a protective substrate action, is usually expected in enzyme systems. Probably other effects of cyanide are concerned than direct enzyme poisoning. From the 8th series definite activation by cyanide of the respiration in glucose was always observed, with aerobic and CN-yeasts; occasional activation in lactate was also found.

Nothing can yet be said on the nature and significance of the blue-fluorescing substance, except that it can be oxidized and reduced, but work is proceeding on this point.

SPECIFIC DISCUSSION.

These results are interesting for several reasons. Strangely enough no clear study of the effects of cyanide on growing yeast has been found in the literature. Neuberg & Perlman [1925] came close to such a study, Patterson [1931] investigated cyanide effects on different yeast preparations, and Meissel [1933] is referred to as having studied the toxic action of cyanide salts on yeast development, chiefly using heavy metal salts, however. Thus the present work opens up a new field for investigation, both with yeast and other organisms.

Points of special interest are (1) the increase in flavin in CN-yeast, (2) the relative insensitivity to CN of the respiration in CN-yeast, (3) the abolition of the Pasteur effect and (4) the development of a new type of yeast with perhaps a new respiratory pigment. In any case it is clear that cyanide-yeast represents a type distinct from either aerobic (baker's) or anaerobic (bottom) yeast.

The suggestion [Pett, 1935, 3] that the respiration of CN-yeast might be carried on by the increased flavin has been criticized by Torres [1935] on the basis of Warburg's "Wechselzahl" calculations. The fact remains that CN-yeast has 2 to 6 times more flavin than ordinary yeast and a respiration largely insensitive to cyanide.

Krah [1930] and Eichholtz [1930] had suggested that the Pasteur effect is connected with the poisoning of a ferro-catalyst so it was interesting to find that the effect has disappeared from CN-yeast. Since it reappears, even weakly, on seeding into medium followed by aerobic growth, it must be concluded that the mechanism was not destroyed. It would be interesting to see the effect of growing yeast in ethyl carbylamine which Warburg [1926] showed to inhibit the Pasteur effect without affecting respiration or fermentation. More dilute cyanide concentrations might accomplish the same thing, as preliminary experiments have indicated.

More work is necessary on the effect of prolonged subculturing in cyanide before the significance of the observations is clear, but the indication is that some profound disturbance of the yeast has been effected and may result in a distinct type.

General discussion.

The researches of Warburg have led to the conception of cyanide as a poison of an iron-containing haem-like enzyme which is essential for the transfer of oxygen in all cells. The early work even referred to "das Atmungsferment" as though only one enzyme was responsible, though this idea has been relaxed.

It has been generally insisted that a cell's respiration must be largely inhibited by cyanide. Other results have appeared from time to time. Dixon & Elliott [1929] pointed out that while yeast respiration in general is inhibited by CN to an extent of 80 or 90 %, sometimes this did not result, and the respirations of various animal tissues were by no means totally, or even largely, inhibited by cyanide. Again Rosenthal [1931], Banga *et al.* [1931] and Shoup & Boykin [1931] found various cells and tissues partly or largely insensitive to cyanide. Of special interest here is the work of Elvehjem [1931] on cultured yeast. The studies of Frei *et al.* [1934] and of Yamagutchi [1934] have made it clear that the respiration of many, if not most, bacterial species is not very sensitive to cyanide, whether they contain cytochrome and its oxidase or not. Other possible effects of cyanide were, as usual, not studied. Occasional observations of activation by cyanide have been made. Thus Reynolds [1924] showed an increase in growth of *Fusarium*; Hanes & Barker [1931] observed an increase in the respiration of potato tubers; Tomkins [1932] observed an ultimate increased growth of moulds due to cyanide; and the respiration of algae [Watanabe, 1932] and of mammalian tissues [Kisch, 1933] has been increased by cyanide. Recently Lindahl & Örström [1936] reported an activation of top yeast respiration, which has always been the best subject on which to show inhibition. As to other effects of cyanide on cells few results have been reported. Resnichenko [1928] pointed out the importance of *p*H and permeability in cyanide effects, and Brinley [1928] suggested that one effect of cyanide may be to alter cell membranes and protoplasmic viscosity.

The present results, in which yeast has been actually grown in media containing cyanide, thereby effecting changes in the yeast, coupled with the above observations of others, make it imperative that the effects of cyanide on cells be considered from a broader view than heretofore. Especially must be revised the narrow view that respiration is invariably CN-sensitive and that this sensitivity is a result of specific action on certain enzyme systems, remote from all other possible effects. The importance of different effects from different cyanide concentrations must be recognized—indicating different actions, and above all the possibility of effects on permeability and metabolic processes which may or may not be reflected in the respiration, because the action is not solely, if at all, on the respiratory enzyme systems.

SUMMARY.

The observation that yeast can be grown in cyanide has been extended. Controls aerated by pure air (like baker's yeast), by nitrogen (corresponding to bottom yeast), and by nitrogen plus cyanide have been studied simultaneously. The respiration, fermentation, etc., of the different yeasts have been determined, and cyanide-yeast is shown to be a distinct type.

Cyanide-yeast showed the following properties, among others:

1. The flavin, present as flavoprotein, was consistently 2 to 6 times higher than in controls.
2. The respiration with or without substrate was largely insensitive to *M*/600 cyanide.
3. The aerobic fermentation was higher than the fermentation of aerobic yeast whether determined aerobically or in nitrogen.
4. The Pasteur effect was not demonstrable—the anaerobic fermentation was no higher than the aerobic.

The effect of prolonged subculturing in cyanide indicates the possibility of developing a new type of yeast, containing no flavin but rather a blue-fluorescing pigment.

In the general discussion it is pointed out that the effects of cyanide on cells must now be considered from a broader view, recognizing that cyanide does not always inhibit, and that several effects probably exist.

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CCV. SOME RELATIONS BETWEEN ASCORBIC ACID AND GLUTATHIONE.

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ASCORBIC acid and glutathione are the most conspicuous and, so far as is at present known, the most active reducing substances in living tissues. In spite of the fact that their fundamental constitutions and physiological functions are so different they have certain qualities in common. Both agree for instance in the circumstance that though their reduced and oxidized forms may co-exist in a tissue, they form redox systems which are not thermodynamically but only chemically reversible. Though in a given case the function of either may be specific, as is that of glutathione in the glyoxalase system, other systems are known in which one can replace the other, probably because in such cases reducing power alone determines their influence. Doubtless other more specific kinetic functions may be revealed in the future. Meanwhile the question arises whether, as reducing substances with different redox potentials, they can exert combined activities, or display interrelations of importance. This paper deals with their mutual relations as displayed in particular circumstances.

I. THE SYSTEM HEXOXIDASE-ASCORBIC ACID-GLUTATHIONE *IN VITRO*.

Szent-Györgyi [1931] described a plant enzyme which oxidizes ascorbic (hexuronic) acid reversibly. He called it "hexoxidase" and though the name of its substrate has been changed it will be convenient, in this paper at least, to retain for the enzyme its original designation. Szent-Györgyi had already shown [1928] that ascorbic (hexuronic) acid was oxidized by metal catalysis but catalysis by the enzyme is much more efficient. In commenting on the specificity of the latter he remarked "also glutathione remains unoxidized in the presence of hexoxidase. If, however, hexuronic acid is present the glutathione is oxidized; the hexuronic acid plays the role of catalyst; it is oxidized by the enzyme and reduced by glutathione". Szent-Györgyi was therefore at that time conversant with the essential relations which hold in the system now to be described; but his studies went no further than what is expressed in the above qualitative statement. Meanwhile Mawson [1935] has studied the effect of tissue extracts in protecting ascorbic from oxidation and found that part, though by no means all, of their influence is due to their content of glutathione (GSH), a conclusion to which de Caro & Giani [1934] had also come. Kellie & Zilva [1934] showed that tissue extracts inhibit oxidation by copper. Bersin *et al.* [1935] have also shown that the autoxidation of ascorbic acid *in vitro* is inhibited by (in their experiments relatively large amounts of) GSH.

A closer and quantitative study of the system ascorbic acid-glutathione-enzyme has shown that it is one of much interest because of the definite manner in which it allows mutual relations to be displayed.

Methods.

Szent-Györgyi obtained the enzyme from the expressed juice of cabbage leaves. This source we have also used, but we have found the juice from the florets of the cauliflower and the central white stalks which carry them especially convenient, owing to its freedom from chlorophyll and to the much smaller amounts of soluble substances contained in it. The enzyme seems to be present in high concentration in all varieties of *Brassica*, and is certainly widely distributed in other species. To determine its distribution more exactly would be of interest. Zilva [1934] decided, doubtless rightly, that the rapid disappearance of ascorbic acid from apples was probably due to this enzyme. Tauber *et al.* [1935] prepared and studied an enzyme from *Curcubita maxima* which oxidizes ascorbic acid but which, though it would seem on insufficient grounds, they decided was different from Szent-Györgyi's hexoxidase.

Szent-Györgyi concentrated his enzyme preparation by precipitation with ammonium sulphate, and this method we have occasionally followed. In the majority of our experiments however we have used the expressed juice itself. If this contains oxidizing agents other than the hexoxidase they certainly do not exercise any influence on the system as studied. It was usually prepared from cauliflowers in bloom, being expressed from the plant tissues by the use of a screw press with a linen filter, and afterwards centrifuged. It is then clear, nearly colourless and contains a concentration of the enzyme which, though always high, varies somewhat with the source. It is always higher in the fresher home-grown plants than in those imported. In the present research it has not seemed necessary to standardize the strength of enzyme preparations used. In every case where the purpose of an experiment has made equality of concentrations necessary the same preparation has of course been used. In order that the relations to be discussed should be clearly shown it is necessary, as will immediately be understood, that the juice or enzyme preparation employed should be entirely free from ascorbic acid. 48 hours' dialysis in a cellophane dialysing tube with running water secures this freedom.

To follow the progress of concentration changes in the system when aerobic conditions were required solutions of the reactants were uniformly shaken in a series of open Erlenmeyer flasks, the contents of each flask being at first identical. At suitable intervals one of the flasks was removed, further change being immediately stopped by the addition of trichloroacetic acid, and the contents were titrated. The concentrations of ascorbic acid and glutathione (GSH) present at each stage of the experiment were determined by titrating half of the solution with $N/100$ iodine in the presence of potassium iodide and the other half with Tillman's reagent, using a micro-method [Birch *et al.*, 1933; Hopkins *et al.*, 1935]. The iodine value of the ascorbic acid so estimated was calculated and deducted from the total iodine figure to give the figure for glutathione. When these two substances are in solution alone, unassociated with other reducing substances, much experience has shown that this method gives results which are completely accurate.

EXPERIMENTAL RESULTS.

To save space all the results obtained are displayed in the figures without numerical tables to correspond. For convenience the concentrations of the ascorbic acid and glutathione are given in terms of ml. of $N/100$ iodine. This makes easier their presentation together in a single figure. It may be useful to recall that to reduce a molecule of iodine two molecules of glutathione are required, but only one of ascorbic acid, and that 1 ml. of *N*/100 iodine corresponds to 3·07 mg. of the former and 0·88 mg. of the latter.

The oxidation of ascorbic acid itself as catalysed by the enzyme may be first illustrated. Fig. 1 shows the characteristic linear course of the reaction; its velocity being proportional to the enzyme concentration. Fig. 2 shows the marked effect of *p*H upon the velocity. It is much greater at *p*H 6·0 than at 7·4, the former being near to the *p*H of the expressed juice.

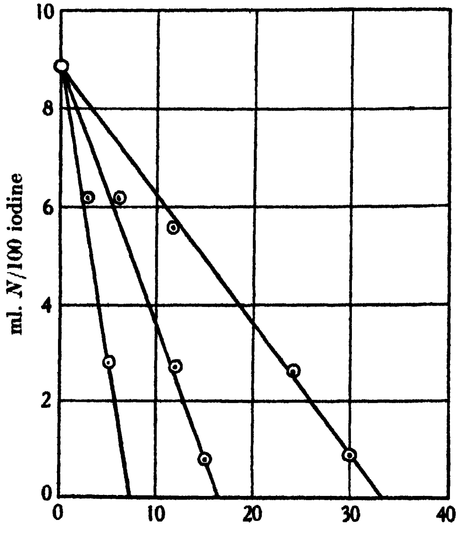

Fig. 1.

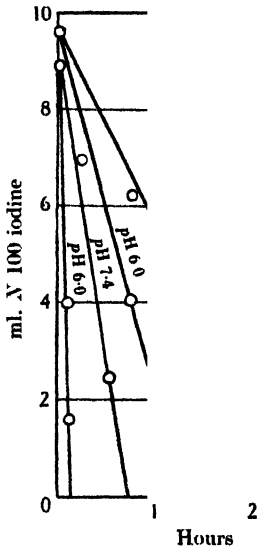

Fig. 2.

Fig. 1. Shows the course of oxidation of ascorbic acid (8 mg.) at *p*H 6·0 and 18° with 1, 2 and 4 ml. of the same enzyme preparation. The velocity is seen to be closely proportional to the enzyme concentration.

Fig. 2. Shows effect of *p*H on the velocity of oxidation by the enzyme in high and lower concentrations. Temp. 20°.

Fig. 3, which presents at a glance the relations which are always displayed in the system studied, has almost the appearance of a diagram, but is actually constructed from experimental data plotted in the ordinary way. In the experiments yielding the results which this figure summarizes the enzyme preparation was thoroughly dialysed juice and was identical and at the same concentration in each of the three experiments involved. They were carried out at *p*H 7·4, and the time scale extended in order that the relations should be clearly shown. The ascorbic acid and glutathione were present throughout in the proportion of approximately 1 mol. of the former to 2 of the latter. (If exactly in these proportions the resulting oxidation curves would overlap.)

The sloping dotted line shows the course of oxidation of ascorbic acid when alone with the enzyme. The horizontal continuous line shows the behaviour of glutathione when alone with the enzyme; it was unaffected. When however both substances were present together the ascorbic acid was wholly protected from oxidation (horizontal dotted line) whilst the glutathione was oxidized at exactly the same rate as was ascorbic acid when alone (sloping continuous line). It wil be seen that when the glutathione was very nearly all oxidized, but only then, the oxidation of ascorbic acid promptly began. The completely linear course of both the direct oxidation of ascorbic acid and the indirect oxidation of glutathione is noteworthy, and also the circumstance that the courses are exactly parallel. These relations are always reproducible in the system, remaining essentially the same when the relative concentrations of the reactants are varied widely. Fig. 4 for instance shows the results of two experiments in which the enzyme concentration was higher than in those of Fig. 3 (the time scale being therefore shortened in the figure). In case *A* the concentration of ascorbic acid was twice that in case *B*, whilst the concentration of glutathione was the same. In each case it will be seen that the protection of the former lasted till the latter was nearly all oxidized.

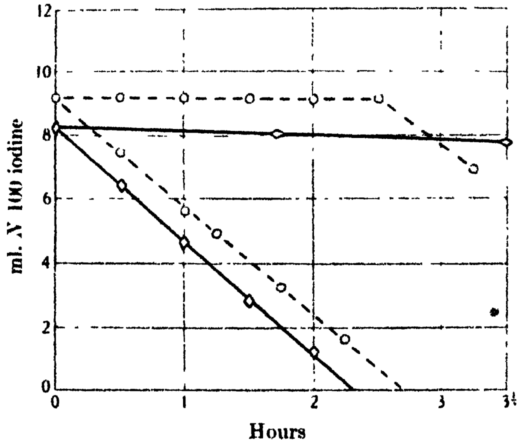

Fig. 3.

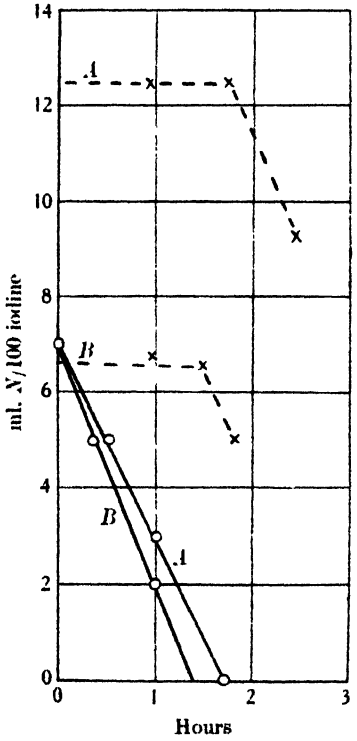

Fig. 4.

Fig. 3. Shows the influence of the oxidase in identical concentrations on ascorbic acid (broken lines) and glutathione (continuous lines), when alone and when together. In each case 1 ml. of dialysed juice in 10 ml. of solution buffered at *p*H 7·4. Temp. 18°. For discussion see text.

Fig. 4. Course of oxidations with both components initially present together. In case *A* the concentration of ascorbic acid was approximately twice that in *B*; that of GSH the same in each case. *p*H 7·4. Temp. 18°.

The relations thus brought to light by the use of iodine titrations were further illustrated by measurements of oxygen uptake in the Barcroft differential manometer, the various adjustments in the instrument being made as usual.

Fig. 5 shows results which are typical of several obtained. A solution containing 2·64 mg. of ascorbic acid and 9·9 mg. of GSH (1 : 2 mol. approx.) buffered with phosphate to *p*H 6, was placed in a flask of the manometer; the total volume being 3 ml. In a Keilin cup 0·33 ml. of undialysed juice expressed from cauliflowers was placed, and this was tipped into the solution only after the apparatus was equilibrated in the bath (temp. 18°). Side by side with this a second manometer was shaken, containing, in precisely similar circumstances, 2·64 mg. of ascorbic acid alone. The juice was an active one and the *p*H near the optimum for the enzyme. The oxygen uptake was therefore rapid. It will be seen that its course was, as always, strictly linear, and its earlier stage, when the glutathione was alone being oxidized, continuous with the later stage of ascorbic acid oxidation. It will be also seen that the linear uptake ceased when the theoretical requirement for the oxidation of both constituents was only slightly exceeded. The oxidation rate of the ascorbic acid, when alone, corresponded so exactly with that of the two components together that the curves completely overlapped.

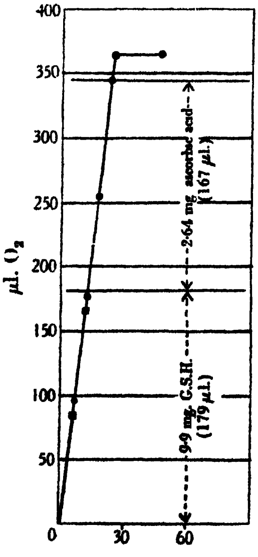

Fig. 5.

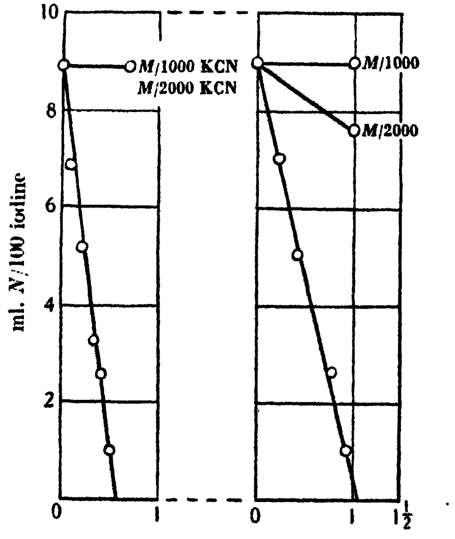

Fig. 6.

Fig. 5. Oxygen uptake of ascorbic acid and glutathione together in solution with the oxidase. The lower horizontal line shows the theoretical uptake for the GSH present and the upper that for both components together. The course of uptake for the ascorbic acid alone in identical conditions was separately determined and two points from the linear course are shown as squares. The velocity was exactly the same as in the case of the mixture.

Fig. 6. Inhibition of the enzyme by cyanide. The right-hand chart shows the effect in the case of the juice; inhibition was complete at *M*/1000 KCN. In the case of an enzyme preparation obtained by precipitation with $(NH)_4SO_4$ *M*/2000 was equally effective; left-hand chart.

Before discussing these results further a few words may be devoted to the nature of the enzyme. Szent-Györgyi found that it was relatively insensitive to cyanide inhibition, and on this circumstance, together with the fact that the rate of oxidation of ascorbic acid under its influence varied so slightly with varying concentrations of the substrate, he based the opinion that the mechanism of its action must be different from that of all other oxidases. We have not been able to confirm the insensitivity to cyanide; $M/1000$ KCN we have found to inhibit oxidation by the juice completely (Fig. 6). In the case of an enzyme preparation obtained by precipitation with ammonium sulphate inhibition was complete at $M/2000$. The linear rate of oxidation, showing no loss of velocity with the fall in substrate concentration is not out of harmony with the Michaelis-Menten theory. In the case of the hexoxidase, however, it is somewhat remarkable that the velocity remains constant until the substrate is so nearly oxidized. Its kinetics would doubtless repay a thorough study. On the basis of the theory the Michaelis constant of the enzyme must be very small, and it is perhaps necessary to assume that it activates both the reduced and oxidized molecules of ascorbic acid. With regard to the nature of the oxidation suffered by the ascorbic acid molecule, although it has been suggested that this involves only the removal of two hydrogen atoms from the carbons which in the original molecule are united by a double bond, the classical work of the Birmingham School makes it almost certain that it involves rather the addition of two hydroxyl groups to those carbon atoms [Herbert *et al.* 1933]. The distinction however is perhaps unimportant as the adjacent carbonyl groups left by the removal of the two hydrogens would in any case almost certainly suffer hydration. The enzyme is quite unaffected by phenylurethane as we have found and, as Szent-Györgyi found, it does not accelerate the reduction of methylene blue by ascorbic acid. This and its cyanide-sensitiveness bring it under the accepted definition of an oxidase. Among oxidases it seems to be highly specialized in its activity, as Szent-Györgyi. and, since, Tauber *et al.* [1935] have found.

We have seen that the outstanding feature of the system formed when ascorbic acid and glutathione are together under the influence of hexoxidase is the complete protection from oxidation of the specific substrate and the indirect oxidation of the constituent which when alone is unaffected by the enzyme. There can be no doubt that this follows from the circumstance that hydrogen is transferred from two molecules of glutathione to each activated molecule of ascorbic acid, maintaining it thus in its reduced form. In explanation of the steady maintenance displayed so long as even very small amounts of glutathione remain in the system, it would seem that the oxidation of ascorbic acid should prove to be a slower process than its reduction. We have endeavoured to test this point experimentally.

As others have found, the product of the reversible oxidation of ascorbic acid is remarkably unstable and difficult to isolate. On the other hand solutions prepared by oxidation with iodine for instance, which was first used by Szent-Györgyi himself, have the disadvantage that when their *p*H is adjusted a relatively large amount of iodide must be present, making such solutions unsatisfactory for enzyme studies. To follow the rate of reduction by glutathione we proceeded therefore as follows.

Oxidation by the enzyme was first carried out by placing equal amounts of ascorbic acid in buffered solution at known *p*H, in each of a series of Thunberg tubes provided with stoppers carrying a bulb turned downwards. An equal amount of enzyme was measured into each of the tubes, and all, at this stage left open to the air, were uniformly shaken by a motor. The course of oxidation was followed as usual by removing individual tubes at intervals and determining therein the remaining concentration of reduced ascorbic acid. When reversible oxidation was just completed (*vide infra*) a known amount of glutathione in neutralized solution was measured into the bulbs and the tubes stoppered. All were fully evacuated and the glutathione then mixed with the main solution. The tubes were returned to the shaker and the increasing concentration of reduced ascorbic acid followed by successive estimations. An advantage of this method of experiment is that each sample remains in the same vessel during the processes and the enzyme present is the same throughout. With regard to the preliminary oxidation it is important to realize that though the enzyme first oxidizes the ascorbic acid reversibly, if its action is allowed to continue long enough irreversible oxidation may follow. Fortunately however in solutions somewhat on the acid side of neutrality, e.g. at *p*H 6, the former process is completed before the latter begins. Moreover, since oxidation follows a linear course so exactly, it is easy after two successive estimations have been made in the series to follow the slope thus established down to the base line and thus learn the moment when the reversible oxidation will be complete but not exceeded. The information is thus given with remarkable accuracy. In the experiments under description the tubes were therefore removed for the reduction process just before the time so indicated, and the amount of reduced ascorbic acid ultimately restored in the reversal process was found to correspond, often exactly, with the amount present before the process of oxidation began.

The results of a typical experiment of the above sort are demonstrated in Fig. 7, in which it should be noted the time scale is in minutes, not hours. In this experiment the reactants were employed in the proportion of approximately 1 mol. of ascorbic acid to 4 mol. of glutathione. When aeration began 7·8 mg. of ascorbic acid were present in each tube and when the oxidation was completed 60 mg. of glutathione were added for the reduction process. The solutions were buffered with phosphate buffer at *p*H 6, the total volume being in each flask 10 ml. during the oxidation and 12 ml. during reduction. The enzyme was that contained in 1 ml. of undialysed fresh juice. Temperature 21°.

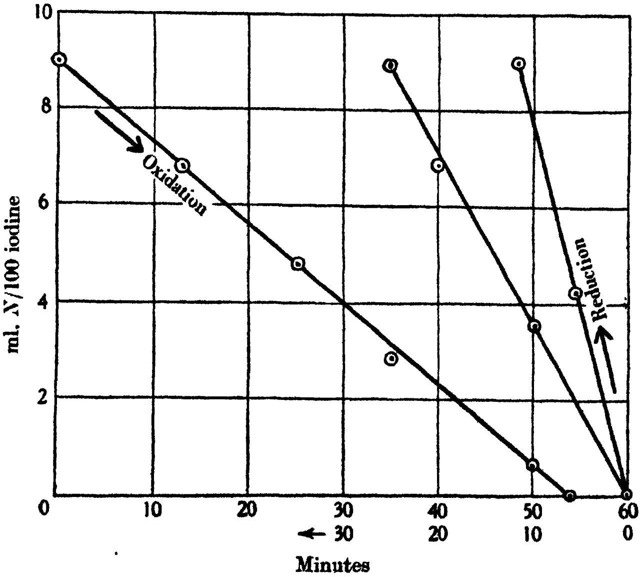

Fig. 7. Enzymic oxidation of ascorbic acid followed by reduction. The latter was induced by adding GSH to the system at the moment when reversible oxidation was complete. The right-hand line shows the rate of reduction in anaerobic conditions. It was five times as great as the rate of oxidation. In this experiment undialysed juice was used for catalysis (see text).

In the figure the left-hand line shows the course of the preliminary oxidation of the ascorbic acid when alone, and that farthest to the right shows the (equally linear) course of the anaerobic reduction in the presence of the glutathione. It will be seen that the former occupied 58 min. and the latter (the original concentration of reduced ascorbic acid being exactly restored) 12 min., the velocity of the reduction at *p*H 6 being thus nearly five times that of the oxidation. The middle line of Fig. 7 shows merely the slower reduction which occurred when tubes containing the enzyme-oxidized ascorbic acid and the added glutathione stood open to the air without shaking.

These results were obtained, as stated, at *p*H 6 and have been frequently repeated. It was found difficult to reproduce them satisfactorily at 7·4, for some at least of the irreversible product then appears early in the course of oxidation. That, in the absence of the enzyme, slow irreversible oxidation by molecular oxygen occurs at *p*H 7·4 and upwards is well known [Barron *et al.* 1936].

A variation in experiments of the kind last described may be looked upon as supplementing those illustrated in Figs. 3 and 4. The ascorbic acid was first oxidized by the enzyme as before, but the subsequent reduction by GSH, instead of being anaerobic, was allowed to proceed while shaking the solution in open flasks was continued. The results of one such experiment may be briefly reported.

In this the concentration of the enzyme was made relatively high (2 ml. of active juice in 10 ml. of buffered solution at *p*H 6). At the beginning of the experiment 7·85 mg. of ascorbic acid were present. The reversible oxidation of this took 18 min. for completion. Neutralized GSH in excess (120 mg. in 2 ml. water) was then added, and aeration continued for exactly 18 min. to correspond with the oxidation period; further change was then stopped by the addition of trichloroacetic acid. The oxidation of ascorbic acid during the second period was balanced by reduction and at the end it was fully reduced. Of glutathione 57·3 mg. were found to have been oxidized, while for the reduction of 15·7 (=7·85×2) mg. of ascorbic acid 54·7 mg. are required. As the glutathione preparation may have been to some slight degree autoxidizable, the correspondence is satisfactory. In this case as in experiments of Figs. 3 and 4 the rate of reduction of ascorbic acid, and therefore that of the oxidation of GSH, was of course controlled by the slower reaction of the oxidation of the former.

In the above experiments relatively high concentrations of GSH were employed. With lower concentrations in the same experimental conditions the velocity of reduction becomes less but is always much higher than the oxidation rate. When the concentration is made very low only the initial velocity can be determined as the GSH is of course soon all oxidized.

One important comment remains to be made on the reduction process as it occurs in the system studied. The experiments just described were carried out with fresh juice undialysed. In an experiment carried out with an enzyme preparation made by precipitating the fresh juice with ammonium sulphate reduction also proceeded normally under its influence. On the other hand, an unexplained effect of dialysing the juice itself calls for discussion. In no other type of experiment have we found any difference between the activities of dialysed and undialysed juice. It should be recalled that in those of Figs. 3 and 4, which first revealed the essential relations in the system, dialysed juice was used. These, of course, were aerobic throughout; and it would seem sure that the reduction by GSH which in such conditions maintains the ascorbic acid in the reduced form must be a relatively rapid process. Yet in experiments of the kind last described, in which the oxidation and reduction of ascorbic acid were made separate and successive processes, undialysed and dialysed juices are found to behave differently, though the experiments were carried out on identical lines in each case. Under the influence of the fresh juice the reduction, as we have seen, proceeds rapidly in linear fashion to completion; with the dialysed this is not the case. With the latter the reduction is found to have a high initial velocity but to fall off quickly, ultimately becoming linear at a rate which, instead of being much greater, is less than the rate of oxidation as observed in the first phase of the experiment. This effect of dialysis is not accidental; it has been obtained with juices from different sources all of which induced the rapid reduction when undialysed. It is noteworthy that the curves obtained after dialysis, like those obtained with the fresh juice, were closely similar in all cases though so different from the latter. It should be noted too that the velocity with which any particular specimen of juice oxidizes the ascorbic acid is entirely unaffected by dialysis; the result is therefore certainly not due simply to a falling off in the concentration of the enzyme. The change, moreover, does not occur if a preparation is allowed to stand untreated for as long or longer than the time taken for dialysis. If the removal of a co-enzyme or any equivalent factor were involved, the facts would be of interest as suggesting a difference in the activating mechanisms for oxidation and reduction respectively. We have not been able, however, to re-establish the original activity by adding the dialysate to the dialysed juice. There is again no factor in the undialysed juice which activates glutathione in the sense of increasing its power to reduce methylene blue. At present we have found no explanation for this unexpected effect of dialysis. To discover some method for preparing the oxidase in a form as pure as possible must be the first step towards such an explanation.

It may be noted here that the reduction of ascorbic acid by GSH when uncatalysed is a slow process. One experiment in illustration of this may receive mention. Ascorbic acid was oxidized by quantitative treatment with $N/10$ iodine in potassium iodide and the iodine then removed as lead iodide. The slight excess of lead was removed by adding potassium sulphate and the solution finally adjusted to pH 6. Using this solution the reduction by GSH alone was then followed by the same method as that used in previous experiments (e.g. in that yielding the results summarized in Fig. 7). It was found that in 90 min. only 3·5 mg. of ascorbic acid were reduced whereas in a control experiment, exactly similar, except for the presence of a low concentration of enzyme in the solution, the above amount was reduced in 8 min., and 6·1 mg. in 15 min.

II. THE BEHAVIOUR OF ASCORBIC ACID AND GLUTATHIONE IN CATALYSIS BY COPPER.

Before discussing this it may be recalled that Barron *et al.* [1936] in a careful study of ascorbic acid oxidations showed that it is not autoxidizable at any pH below 7·6, whereas at pH 5·15 for instance its oxidation is actively catalysed by minute amounts of copper. As is well known Meldrum & Dixon [1930] found that pure glutathione is not autoxidizable and is only oxidized by metals when they are in association with an organic factor. They found this to be true of copper as well as of iron. Voegtlin *et al.* [1931] claim on the other hand that, unlike iron, copper itself is a powerful catalyst at any pH which is physiological. Our own experience agrees with that of the former authors though we have found it somewhat more difficult at, say, pH 7·4 to obtain preparations quite as stable to copper as to iron. At pH 6 the same preparations may be stable. In any case the addition of a minute amount of material from a sample of glutathione which has undergone slight decomposition greatly increases the activity of the copper [cf. Meldrum & Dixon, 1930].

In Fig. 8 are summarized the results of an experiment showing the effect of copper upon the oxidation of glutathione and ascorbic acid when separate and when together in solution at *p*H 7·4. The technique of shaking, etc., was the same as in the enzyme experiments. The same concentration of the metal was present in each of the three observations involved. It was added as cupric chloride equivalent to 0·018 mg. of Cu in the 10 ml. of solution employed. The ascorbic acid (4·2 mg.) when alone was rapidly oxidized (lowest line) and the GSH alone (28 mg.) much more slowly (upper continuous line). When both were in solution the ascorbic acid was wholly protected from oxidation (horizontal broken line) its presence however having no effect upon the rate of oxidation of the GSH (upper broken line). This experiment was done with a sample of glutathione which, though stable in the absence of copper, had not been stabilized by Meldrum & Dixon's method. After it had been treated in solution with a large proportion of muscle powder on the lines described by these authors the experiment just described was repeated. The results however were so alike that the curves could be almost superimposed.

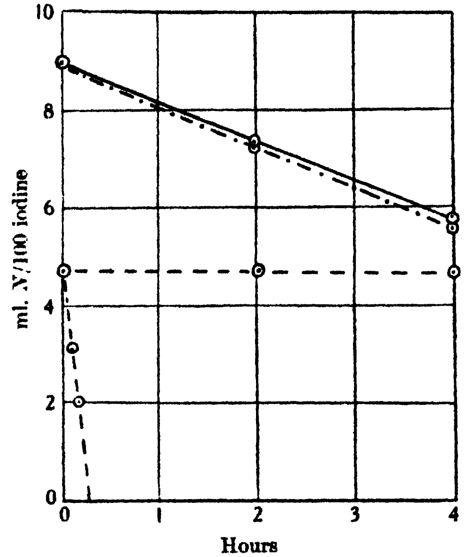

Fig. 8. Behaviour of ascorbic acid and GSH, when alone or together, during catalysis by copper. The lower sloping line shows the initial velocity of ascorbic acid when alone, the horizontal line shows its complete protection in the presence of GSH. The upper lines show the course of GSH oxidation with and without the presence of ascorbic acid. *p*H in each case 7·4. Cu 0·018 mg. in 10 ml. of solution buffered with phosphate.

Fig. 9 refers to an experiment done on precisely similar lines but at *p*H 6. At this *p*H the sample of glutathione employed was stable. The total iodine used in titration remained constant throughout, neither constituent being oxidized (horizontal line). Such a system is inert; one which, as other experiments have shown, takes up no oxygen at all when shaken in a Barcroft manometer.

Clearly, the protection of ascorbic acid by glutathione when the catalyst is a metal must depend upon relations differing entirely from those which secure protection in catalysis by the enzyme. Here there is no question of hydrogen transfer. When the glutathione itself is oxidized in the system the presence of ascorbic acid has no effect upon the velocity of its oxidation (Fig. 10). When, as at *p*H 6, the glutathione is not oxidized the system as just seen is inert. The protection must be due to the fact that the presence of GSH inhibits the influence of the metal on ascorbic acid—a more common type of catalysis inhibition. It must be supposed that although, unlike ascorbic acid, it is itself not oxidized by copper in the absence of an associated organic factor, the thiol form of glutathione (GSH) has a higher affinity for the metal than has ascorbic acid, and prevents effective contact between it and the latter. As might be expected our experiments have shown that the reduction of ascorbic acid by GSH is not catalysed by copper.

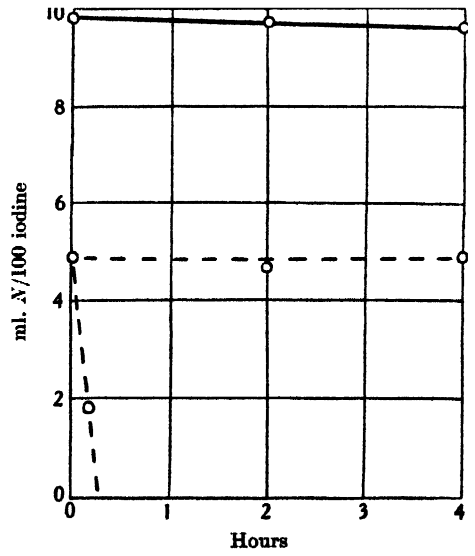

Fig. 9.

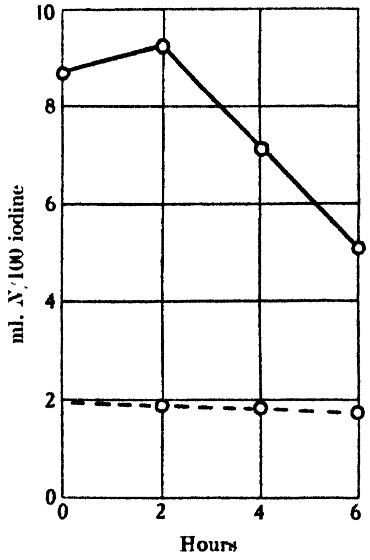

Fig. 10.

Fig. 9. Shows results of an experiment similar in all respects to that of Fig. 8 but carried out at *p*H 6. At this *p*H the GSH was stable and the system with ascorbic acid and enzyme was wholly inert.

Fig. 10. Course of oxidation of GSH (continuous line) and ascorbic acid (dotted line) during the aeration of liver tissue from well-fed rabbits.

III. Some relations in hepatic tissue.

Before dealing with certain oxidation relations between ascorbic acid and glutathione in liver tissue brief reference may be allowed to experiments made six years ago [Hopkins & Elliott, 1931]. These showed that when liver pulp, suspended in mammalian Ringer solution, is aerated by shaking, the course of the oxidation of GSH exhibits characteristically a preliminary period during which reducing processes continue, maintaining or even increasing in fully aerobic conditions the concentration of the GSH. These experiments were made before the importance of ascorbic acid as a reductant was recognized and iodine titration was relied upon for the results obtained. Since then these experiments have been many times repeated, the iodine value of the ascorbic acid present being deducted from the total iodine and the glutathione values obtained by difference plotted as before. The resulting curves display precisely the same form as those published in the paper just quoted.

In other experiments liver slices have been used yielding curves exactly similar in kind. As bearing on the accuracy of such determinations we have found that if hexoxidase be added to a protein-free aqueous extract of the liver or of most other animal tissues it very rapidly reduces the iodine titration figure of the extract to zero. Having regard to the specificity of the action of the enzyme this seems strong evidence in favour of the claim that only ascorbic acid and glutathione are titrated.

Some two years ago we made a number of experiments in which the oxidation rates of glutathione and ascorbic acid in aerated liver tissue were recorded simultaneously. The livers of rabbits in different nutritional conditions were employed. There is at present no evidence that animal tissues contain any enzymic catalyst for the oxidation of either substance. There is no doubt on the other hand that the liver contains sufficient copper to oxidize the ascorbic acid present at a much greater velocity than is observed in the excised tissue, and the intention of the experiments was to discover whether the presence of glutathione plays any part in protecting it from oxidation.

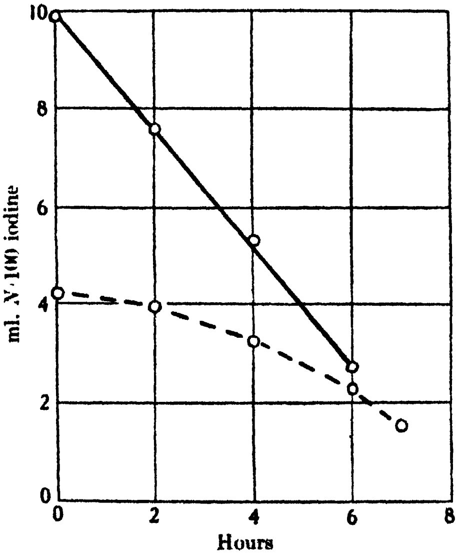

Fig. 11.

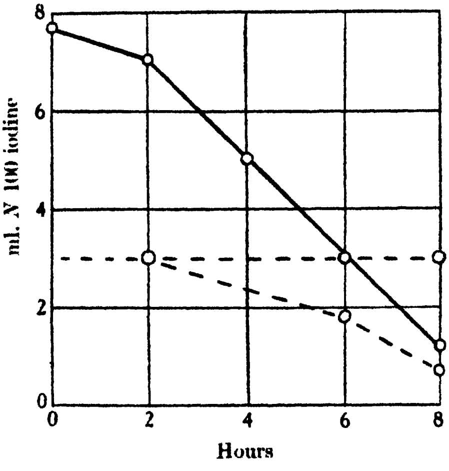

Fig. 12.

Fig. 11. Results of an experiment similar to that of Fig. 10 but obtained from the liver of a rabbit previously deprived of food for 48 hours.

Fig. 12. From an experiment similar to those of Figs. 10 and 11, but showing that ascorbic acid is protected from oxidation if the concentration of GSH is kept high throughout the aeration.

In Figs. 10 and 11 are curves selected from many obtained in the unpublished experiments under reference. In the first the preliminary period of sustained reduction of GSH was well marked and its oxidation relatively slow. The concentration of ascorbic acid is seen to have remained constant. The second shows other conditions. This experiment was done on the liver of a rabbit which had been deprived of food for 48 hours; there was no reduction period, and the oxidation of GSH was rapid. In this case the ascorbic acid was also oxidized with some rapidity. In a general sense the other experiments in the series gave similar indications. Whilst, however, the two curves presented might seem to suggest that the same influences—identical reducing processes for instance—are responsible for controlling the rate of oxidation of both substances, other experiments of the same kind showed that there is too much irregularity in the results for this to be likely. With regard to any possible protective influence on the part of glutathione the curves obtained showed that any observed arrest or slowing of the oxidation of ascorbic acid is not related in any definite way to the absolute or relative concentration of the former existing at the time. If, it is true, the concentration of GSH be maintained at a relatively high value by adding it to the suspension of liver tissue during the course of the oxidations the ascorbic acid may be wholly protected. This is illustrated by the experiment of Fig. 12. Such artificial additions, however, do not reproduce the conditions present in the liver cell itself. It must be admitted that the experiments did not throw any clear light upon the relations of the two substances in the cell. They yield a suggestion that the tripeptide affords some protection to the vitamin but a definite proof or disproof of this calls for experiments of a different kind. The results obtained seem nevertheless to be worthy of record.

Though not strictly germane to this paper some further reference may be here allowed to facts concerning the oxidation of glutathione itself; facts bearing on the question as to how far the substance functions in the transport of hydrogen to oxygen. Experiments are in progress to determine the nature of the reducing systems which, as the experiments of Hopkins & Elliott [1931] showed, survive in excised tissues and, in spite of full aeration of these, delay for a period the oxidation of the GSH. This work is not yet complete and the results will be reported later. It may be stated, however, that the hydrogen donators mainly involved and probably the enzyme or enzymes are special in kind. Mann [1932] showed that the glucose dehydrogenase of Harrison can reduce the peptide, and Meldrum & Tarr [1935] that the isolated dehydrogenase system of Warburg & Christian can do so, even under aerobic conditions. If these systems are both active in the liver (the former certainly is) they are not the sole or most active systems concerned.

We wish, however, to comment here more particularly on a point concerned with the oxidation of GSH. Ogston & Green [1935] found that though rapidly reduced by the glucose and hexosemonophosphate dehydrogenases glutathione does not, when added to these in the presence of their substrates, increase the rate of oxygen uptake; the limiting factor being the rate of its oxidation when reduced. We have found, however, as others have found to be the case with ascorbic acid, that certain factors contained in tissue extracts strongly inhibit even in very low concentration the oxidation of "active" preparations of glutathione; preparations for instance which have stood long enough in aqueous solution to develop the organic factor which Meldrum & Dixon showed to be necessary (together with traces of metal) for oxidation. We have separated from liver a fraction containing a soluble protein (which may have been only an associate of some active inhibitor) of which 5 mg. when added to 25 mg. of activated glutathione in 10 ml. of solution at *p*H 7·4 completely inhibited its oxidation. We have moreover made preparations on orthodox lines of some dehydrogenases and have found that these may contain potent inhibitors of the kind in question. It is necessary therefore to bear these facts in mind in testing the capacity of glutathione to transfer hydrogen from a given dehydrogenase system to oxygen. In our study of this question we have endeavoured to obtain cell-free extracts from the liver capable of oxidizing GSH. It is clear that such extracts while containing the oxidizing agency must be free from the inhibitory factors just mentioned. The following simple and empirical procedure yields preparations which are active. The liver, thoroughly ground up with sand, is extracted with mammalian Ringer solution, maintained at *p*H about 4·5 during the extraction by adding acetic or hydrochloric acid. It is so extracted three or four times and the mixed extracts centrifuged. The supernatant fluid is brought exactly to *p*H 4·5 and then heated to 70–75° and held at that temperature for 3–4 min. It is cooled and the coagulated proteins separated on the centrifuge. The oxidation factors in the extract are not enzymic and it may, if necessary, be concentrated *in vacuo* at low temperatures; not however open on a water-bath. At the above *p*H and in the presence of the electrolytes in the Ringer solution the inhibitory substances are removed with the bulk of the proteins while the oxidizing factors are left largely intact.

The two curves of Fig. 13 show the course of oxidation of GSH when an extract containing it was shaken in a series of open flasks. Oxidation was slow but the experiments were done at room temperature and not at 37°. The characteristic linear course always seen when the liver tissue itself is aerated is not maintained in extracts and must depend on organizing conditions in the cell.

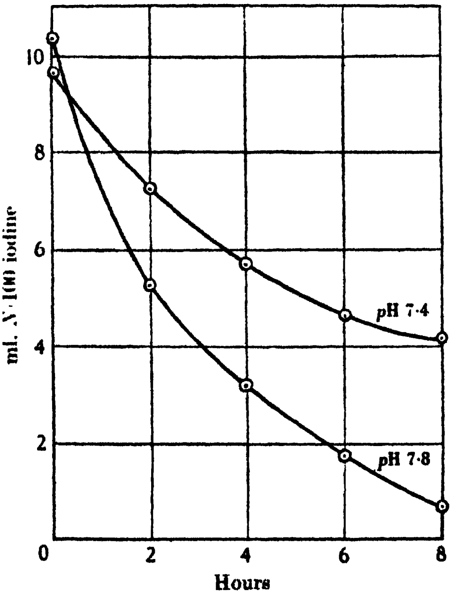

Fig. 13. Oxidation of GSH by cell-free liver extracts at *p*H 7·4 and 7·8. Temp. 18°.

DISCUSSION.

In 1928 Szent-Györgyi when describing for the first time the plant enzyme which specifically catalyses the oxidation of ascorbic acid, pointed out that although the enzyme when alone is without influence on reduced glutathione it oxidizes this when ascorbic acid is present in the system. The latter then "plays the role of a catalyst".

The experiments described in the first section of this paper dealt quantitatively with the system glutathione-ascorbic acid-enzyme. They have shown that so long as it remains in the system GSH completely prevents the oxidation of ascorbic acid. On the other hand, it is itself then oxidized at exactly the same rate as, in similar conditions, ascorbic acid is oxidized when alone. In each case the course of oxidation is linear throughout. Whatever the initial relative concentrations of the components that of ascorbic acid remains constant till the GSH is nearly completely oxidized. It seems clear that during the course of oxidation in the complete system the two hydrogen atoms which, in effect, are transferred from each activated ascorbic acid molecule to oxygen are simultaneously replaced by hydrogen from two molecules of GSH. The rates both of the direct and indirect oxidations are proportional to the enzyme concentrations and are affected alike by variations in *p*H.

The behaviour of the enzyme accords with the definition of an oxidase. It is fully cyanide-sensitive but unaffected by phenylurethane; its presence does not affect the rate at which methylene blue is reduced by ascorbic acid or glutathione. The circumstance that the course of the oxidation of ascorbic acid as catalysed by the enzyme remains linear throughout indicates that it has a high affinity for its specific substrate and that the Michaelis constant must in its case be very small.

To explain the steady maintenance of the reduced form of ascorbic acid in the presence of the oxidase when GSH is also present it seems necessary to assume that reduction of the former by the latter must be a more rapid process than the direct enzymic oxidation. It proved easy to show experimentally that this is the case if the enzyme is supplied as it exists in the undialysed plant juice or when it is contained in preparations made by precipitation from the juice with ammonium sulphate. Ascorbic acid, first oxidized reversibly by the juice or by such preparations, is reduced anaerobically when GSH is then added to the system at a rate which may be five times that of the oxidation. It is a remarkable circumstance however that, though the rate of oxidation induced by any sample of juice is not at all affected by dialysis, the power of the latter to catalyse reduction is much lessened and the course of reduction modified. This phenomenon which is consistently observed is discussed in section I, but no explanation can at present be offered. This, we think, is a reason for a further thorough study of the kinetics of the system. There is perhaps another. If we assume that the oxidized no less than the reduced molecules of ascorbic acid are activated by the enzyme it would seem unnecessary to suppose that the latter exercises any direct influence on the molecules of glutathione. Yet their reducing influence continues unabated when their concentration has become extraordinarily small. It may be justifiable to suppose that they are specifically absorbed and orientated on the enzyme surface and so brought into effective relations with the ascorbic acid molecules although not themselves activated.

The system as studied is an artificial one though its kinetics seem to be of great interest. In any plant tissue which contains glutathione however it might well have physiological functions. Though the presence of glutathione in the growing tissues of *Brassica* is not disproved, we have evidence that it is unlikely, though substances yielding a nitroprusside reaction are present.

The addition of a very minute concentration of ascorbic acid will convert a hexoxidase preparation which is without action on GSH into one which oxidises it, and it is perhaps instructive to realize that if, in the absence of Szent-Györgyi's recognition of his oxidase as one specific for ascorbic acid, a search had been made for an enzyme acting on GSH, it might well have been supposedly found in the juice of *Brassica*. This would have proved to be inactive after purification; a co-enzyme would almost certainly have been then sought and identified with ascorbic acid!

The presence of GSH protects the vitamin from oxidation by copper catalysis no less than by enzymic catalysis. In the former case however the nature of its influence must be wholly different. There can be no question of hydrogen transference. If the glutathione employed in an experiment (owing to its association with small amounts of Meldrum & Dixon's organic factor) is to any degree oxidized by the metal, the presence of ascorbic acid makes no difference to the rate of its oxidation. If, on the other hand, the glutathione is stable the system is completely inert.

The inhibition of the ascorbic acid oxidation seems clearly to be due to a circumstance familiar in other cases of metallic catalysis. The inhibitor—in this case GSH—forms a stable compound with the copper, preventing efficient contact between the metal and its substrate.

It is of interest to find that one cell constituent protects another from oxidation in each of two systems so widely diverse in their mechanisms.

In section III of the paper reference is made to a repetition of the experiments of Hopkins & Elliott [1931] on the oxidation of glutathione in excised liver tissue. The results of these have been confirmed. Other experiments are described in which the oxidation rates of glutathione and ascorbic acid were followed simultaneously. Although they suggest that high concentrations of the former may protect the latter from oxidation in the liver, their general indications are that normally the two substances are oxidized independently, perhaps by different agencies.

SUMMARY.

When ascorbic acid and glutathione are together in the presence of the hexoxidase described by Szent-Györgyi the glutathione wholly protects the vitamin from oxidation, whilst it is itself oxidized at a rate which, with the same concentration of enzyme, is exactly the same as the rate with which ascorbic acid is oxidized when alone. Only when GSH has practically disappeared from the system does the oxidation of ascorbic acid begin.

When ascorbic acid has been reversibly oxidized its reduction by pure glutathione alone is a very slow process; but in the presence of the enzyme (in conditions which are discussed in section I) the reduction may be five times as fast as the oxidation induced by the same concentration of the enzyme.

Glutathione also completely protects ascorbic acid from oxidation by copper catalysis. The mechanism of protection must here be different from that which operates in the case of the enzyme. In the latter it depends upon hydrogen transference, in the former on inhibition of the catalysis.

In the last section of the paper the behaviours of ascorbic acid and glutathione in aerated hepatic tissue are described and discussed.

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CCVI. INVESTIGATIONS OF BLOOD-SERUM LIPOIDS IN CANCER AND OTHER CASES.

I. THE MEAN MOLECULAR WEIGHT OF THE FREE AND COMBINED ACIDS IN THE BLOOD-SERUM LIPOIDS OF CANCER AND OTHER SUBJECTS.

BY HAROLD HAYDEN BARBER.

II. ULTRAVIOLET ABSORPTION MEASUREMENTS.

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I. THE MEAN MOLECULAR WEIGHT OF THE FREE AND COMBINED ACIDS IN THE BLOOD-SERUM LIPOIDS OF CANCER AND OTHER SUBJECTS.

THE work described in the following section is an examination of the method published by von Noël [1931] as a means of diagnosing cancer from the chemical analysis of serum "fat". The underlying principle on which the method is based is the assumption that in cases of cancer, although the depot fat remains of normal composition, the serum fat will show the presence of acids of a mean molecular weight lower than that normally obtained.

The reason put forward in support of this view [Dannmeyer & Seel, 1931] is based on the well-known theory of Warburg *et al.* that there is an increased formation of such substances as lactic, pyruvic and acetoacetic acids as a result of deficient oxidation of carbohydrate in the diseased tissue cells.

The serum lipoids actually employed are those obtainable by extraction of the dried serum with ether followed by extraction with an alcohol-ether solution containing 2 % ethyl alcohol. For the chemical analysis the several ether extracts are mixed with the alcoholic ether extracts and the total residue examined; but in the present work, in those cases where the physical method described in Part II was employed, the alcoholic ether extract was the fraction supplied for the physical examination, and this solution on its return was added to the ether extract for the chemical tests.

The chemical examination consists simply of the determination of the acid value and the ester value of the extract, and from the results of these two determinations the "Krebszahl" [von Noël, 1931] of the specimen is calculated. This "cancer number" is based on the assumption that the normal depot fats have a mean equivalent weight of 280, so that for saponification 1 ml. of 0·1 N alkali solution is equivalent to 28 mg. of fat. If the number of ml. of 0·1 N alkali solution required to saponify 100 mg. of the unknown sample of fat is multiplied by 28, the resulting figure (the Krebszahl) will be very near to 100 if the fat is normal depot fat, but will be greater than 100 if fatty acids of lower molecular weight preponderate.

The Krebszahl is given by the expression

$$\text{Krebszahl} = \frac{100\,(a+b)\,28}{\text{wt. of substance in mg.}},$$

where a = ml. of 0·1 N solution to neutralize free acids and b = ml. of 0·1 N solution to saponify the neutralized fat.

It is also assumed that there is no significant variation in the proportion of the unsaponifiable fraction in the cancer and non-cancer specimens.

An interesting fact not mentioned by von Noël is the simple numerical relationship between the Krebszahl and the ordinary saponification value; the latter, based on a molecular weight for KOH of 56·1, is almost exactly twice (2·004) the former.

From the results of the examination of over 80 blood samples from normal, cancer and other cases, von Noël suggested a critical value of 130 for the Krebszahl, all values above this were claimed to be characteristic of cancer, whereas normal cases and cases of tuberculosis, diabetes, arteriosclerosis, benign tumour etc. gave values below 130.

In order to test this claim more than 40 specimens of blood from cancer and other cases have been examined by the above technique, and in order further to simplify the conditions the majority of the non-cancer cases selected were as near to normal as possible whereas many of the cancer cases were in an advanced stage of disease.

With the exception of the cases A, B and C, the blood specimens were kindly supplied by Mr F. C. Hunt from the Nottingham General Hospital, and with the above exception the details in Table I were also furnished by him.

The specimens were taken by venepuncture practically all after an overnight fast of 15 hours.

EXPERIMENTAL.

The blood specimens were defibrinated by means of a stick in the test-tube used for receiving the sample. They were then transferred to the laboratory, centrifuged in graduated 15 ml. tubes and a definite volume of the serum removed by means of a pipette. To the sample of serum in a small beaker, twice the volume of saturated zinc sulphate solution was added and, after mixing, the contents were poured on to a weighed amount of A.R. anhydrous sodium sulphate contained in a porcelain dish. The weight in grams of sodium sulphate was equal to four times the volume in ml. of the original serum. This proportion was found to cause the resulting paste to solidify when the dish was left overnight in a desiccator.

Next morning the solid mass was transferred to a glazed porcelain mortar and roughly powdered by hand. The pestle was then clamped in an automatic grinding device, electrically driven, which ground the mass to a fine powder in 30 min. The powder was transferred to a 750 ml. conical separating funnel fitted with a plug of fat-free cotton wool in the apex, and the contents were covered with dry ether and left overnight. (The ether used throughout this work was of B.P. (sp. gr. 0·720) quality which had been dried over sodium wire and distilled from further sodium.) The following morning the solution was run out of the funnel into a flask, the last runnings being blown out by means of dry oxygen-free compressed nitrogen. The pasty residue in the funnel was then shaken with a second quantity of ether which was immediately blown out into the first extract. The residue was dug out of the funnel into the mortar, a little more (about 10 %) anhydrous sodium sulphate added, and the whole reground for half an hour. The powder was then returned to the funnel, covered with ether and again allowed to stand overnight. Next day the previous day's procedure was repeated. On the third morning of the ether extractions the first ether extract only was added to all the previous extracts.

Thus if $a \equiv$ addition of sodium sulphate and grinding and $b \equiv$ addition of ether and running and blowing off, then the total steps are

$$a, b, b; \quad a, b, b; \quad a, b.$$

The total ether extract thus obtained measured about 350–400 ml.

The mass in the funnel was then treated with an alcoholic ether mixture containing 2 % of ethyl alcohol according to the following scheme:

$$c; \quad a, c, c; \quad a, c, c,$$

where $c \equiv$ addition of alcoholic ether followed by running and blowing off, the funnel again being left overnight after each further grinding.

The total alcoholic ether extract, of about the same volume as the total ether extract, was distilled to about 25 ml., and in the cases where the absorption spectrum was investigated was passed on to L. A. Woodward for the work described in Part II of this paper. After its return the alcoholic ether extract was added to the similarly concentrated ether extract and the total mixture used for the chemical examination. This procedure was followed in all cases whether the physical examination was carried out or not.

In von Noël's original paper [1931] the steps in the extraction process are not very clearly described and the above method of procedure is that given in a private communication from him.

The total extract, concentrated to a volume of about 15 ml., was then filtered through a small paper into a 50 ml. tared Erlenmeyer flask and the solvent removed by blowing dry oxygen-free nitrogen on to the surface of the filtrate. The flask was then heated to constant weight in a vacuum drying-oven at 37°. All weighings were carried out to five places of decimals in grams on a Kuhlmann microbalance. The residue was obtained as a colourless or very pale yellow crystalline mass.

The acid and ester values were then determined in the following manner.

The fat was quantitatively dissolved in 15 ml. of a neutralized ether-alcohol solution consisting of 2 volumes of alcohol to 1 volume of ether, immediately transferred to a 100 ml. flask and directly titrated with $N/10$ sodium hydroxide solution from a microburette using phenolphthalein as indicator. The ester value was determined on the neutralized mixture by adding 20 ml. of an ether-alcohol potassium hydroxide solution freshly prepared in the following way. To 20 ml. of $N/2$ alcoholic potassium hydroxide solution were added 60 ml. of absolute alcohol and 40 ml. of dry ether giving a solution of $N/12$ concentration. The saponification was carried out by heating the flask for half an hour on an electrically heated water-bath with frequent shaking and, after cooling, by back-titrating the contents with $N/10$ hydrochloric acid solution. Two blank titrations were carried out, each on 15 ml. of the neutral ether-alcohol solution heated simultaneously with the samples under test.

The results obtained from 41 specimens are given in Table I.

Table I.

Ref. no.	Age	Sex	Clinical diagnosis	Cancer (C), non-cancer (N)	Vol. of serum ml.	Wt. of "Fat" mg.	Vol. of $N/10$ NaOH to neutralize ml.	Vol. of $N/10$ KOH to saponify after neutralization ml.	"Krebszahl"
1	22	F	Chronic appendicitis	N	14·5	86·69	7·58	1·26	285
A	60	M	Carcinoma of rectum with colostomy. Columnar type	C	8·5	77·72	7·32	0·60	285
B	47	M	Bronchitis and asthma	N	8·5	44·85	3·08	0·50	224
C	24	M	Neurasthenia	N	10·5	53·20	5·23	0·55	304
15	63	F	Malignant left ovarian cyst. Secondary deposits in liver, ascites	C	5·0	57·07	4·20	0·57	234
16	20	F	Acute appendicitis	N	6·5	39·05	2·97	0·33	165
19	48	M	Carcinoma of colon	C	10·5	80·65	6·35	0·93	253
20	46	M	Acute appendicitis	N	8·5	91·00	5·43	1·33	208
21	18	M	T.B. pleural effusion	N	8·3	56·46	5·02	1·05	301
22	43	M	Gastric ulcer	N	6·1	68·10	4·76	1·40	253
23	52	M	Functional dyspepsia	N	7·6	47·20	2·76	1·07	227
24	62	M	Carcinoma of rectum	C	6·4	57·10	4·84	1·09	291
25	64	M	Carcinoma of rectum	C	9·5	28·00	2·50	0·39	289
26	70	M	Carcinoma of colon	C	8·8	81·95	7·75	1·03	300
27	53	F	Advanced carcinoma of cervix uteri	C	8·5	41·34	1·91	0·93	192
28	57	F	Columnar celled carcinoma of rectum	C	7·0	51·60	1·48	1·32	152
30	24	M	Hydrocele	N	6·8	33·00	2·12	0·88	255
31	41	M	Acute nephritis	N	8·4	42·20	0·98	1·02	133
32	57	M	? Gastric ulcer	N	12·2	31·80	0·80	0·99	296
33	70	M	Epithelioma of tongue	C	7·0	21·61	0·87	0·91	231
34	69	F	Epithelioma of tongue	C	7·4	31·60	1·54	1·37	258
35	40	F	Carcinoma. Radical amputation right breast	C	9·5	32·39	0·62	0·91	132
36	19	M	Acute mastoiditis	N	9·5	28·37	1·08	1·36	241
37	45	F	?Left hemiplegia. Cerebral tumour	N	7·5	41·10	1·02	1·44	168
38	49	F	Large inoperable carcinoma of left breast. Blood sample, 27. x. 33	C	7·5	38·42	0·77	2·60	246
39	39	F	Caesarian section	N	4·0	14·45	0·56	0·42	190
40	59	F	Fracture dislocation of head of left humerus	N	4·0	16·90	0·65	0·58	204
51	48	F	Advanced carcinoma of cervix uteri	C	13·0	34·00	0·79	1·10	156
52	See No. 38		Now large actively growing mass of carcinoma of left breast. Radium in Oct. 1933 (sample, 20. ii. 34)	C	8·0	22·51	0·46	0·63	136
53	46	F	Acute pyelitis	N	9·0	39·40	0·82	1·48	164
54	38	F	Concussion and abrasions 8 days previously	N	10·0	19·09	0·24	0·53	113
55	30	F	? Cholecystitis	N	7·0	20·49	0·58	0·66	169
56	36	F	Carcinoma of cervix uteri rapidly growing	C	7·6	22·11	0·29	1·03	167
57	16	M	Acute appendicitis	N	7·0	13·92	0·28	0·35	127
58	62	M	Epithelioma of tongue	C	5·6	10·18	0·27	0·26	146
59	35	M	? Malignant disease of left lung	C	7·8	29·66	0·55	1·31	176
60	24	M	Calculi in left ureter	N	6·5	17·28	0·32	0·42	120
61	60	M	Advanced carcinoma of colon	C	7·5	14·65	0·41	0·34	143
62	63	M	Cerebral haemorrhage. W.R. neg.	N	7·7	23·77	0·31	0·42	86
63	21	M	Chronic suppurative otitis media	N	6·6	14·28	0·35	0·34	135
64	60	M	Epithelioma of palate	C	6·5	22·99	0·37	0·51	107

Average Krebszahl for 19 cases of Cancer = 205.
Average Krebszahl for 22 cases other than Cancer = 199.

DISCUSSION.

Two facts which emerge from the foregoing tables of results are (*a*) The "Krebszahlen" have a somewhat higher average value than the figures given by von Noël [1931] and (*b*) there is no indication, from the cases examined, of a higher "Krebszahl" generally characterizing cancer.

The saponification value of the serum lipoids is an analytical figure of such a general nature and the lipoid fraction itself under the conditions of isolation must of necessity contain so many different substances ranging from free acids of 3 or 4 carbon atoms to complex glycerides and cholesteryl esters, that the figures obtained can reveal only a very general average composition. The fact that the saponification value of such a complex mixture does not show any significant differences in value characteristic of one single pathological condition is not therefore remarkable. In a further private communication von Noël reports that he has personally found that an increased amount of bile acids in the blood stream tends to give a false value on account of their high molecular weight.

It would seem too that even in cases where super-normal amounts of such relatively strong acids as lactic or acetoacetic acid are formed, if the acidosis is compensated the technique under discussion would not reveal their presence in any quantitative manner. An alternative possibility that the acids in question could be present as esters, thus revealing themselves by an enhanced ester value, seems somewhat improbable.

It is suggested therefore, on purely theoretical grounds, that the detection and estimation of the relatively strongly dissociated acids generally considered to be characteristic of the abnormal glycolysis of pathogenic tumours, is far from quantitative when attempted by measurement of the acid or saponification value of serum lipoids.

II. ULTRAVIOLET ABSORPTION MEASUREMENTS.

Introduction.

The measurements described in this Part were made with the object of testing the claim of certain previous workers [Dannmeyer *et al.*, 1931] to have obtained a method of diagnosing cancer by observation of the shape of the ultraviolet absorption curve of an extract from blood serum. The procedure of these workers was therefore followed as closely as possible. The work is intimately connected with that described in Part I of this paper, since the explanation put forward by the previous workers for the reported differences between the absorption curves of cancer and non-cancer cases is similar to that for the differences between the chemical "Krebszahlen", reference to which has been made above. Actually, however, the chemical results of Part I lend no support to the corresponding conclusions of the previous workers; but this fact does not preclude the possibility that absorption measurements, which are inherently capable of giving individual information about the different species present rather than an average result for the whole system, might provide a reliable diagnostic criterion.

Experimental.

The measurements were made with a special type of photoelectric spectrophotometer designed and constructed by the writer for the purpose. A description of this instrument and method and of its accuracy has already been published elsewhere [Woodward, 1934]. As in the measurements of the previous workers, a double quartz monochromator was used, so that the illumination of the liquid under investigation was very nearly monochromatic. The source of light was a mercury vapour arc and determinations were made at the following wave-lengths: 313·0, 302·3, 296·8, 289·4, 280·2, 275·6, 265·3, 253·7, 248·1, 240·0 mμ. Where the mercury line in question consists of a close assemblage of components not separable by the monochromator, the wave-length given is an average reckoned with due regard to the relative intensities of the components. Under the conditions of experiment the maximum error in the determination of extinction coefficients is about 0·5 %; except for the highest and lowest values, for which it is about 1 %.

Owing to the volatility of the solvent (ether + 2 % alcohol) difficulties were encountered in using absorption vessels of the customary type, i.e. consisting of a tubular glass distance-piece with optically worked ends upon which are pressed quartz end-plates. With such vessels it was found impossible to prevent the formation in the liquid of a bubble, the presence of which gave rise to errors in the measurements. The trouble was overcome by using fused silica one-piece absorption cells (Hilger Type D) in which the plane end-plates are adhesed on to the tubular part of the cell by a special process without cements. These vessels, the filling tubes of which were closed during the experiments by means of cork stoppers previously washed with ether, proved entirely satisfactory. Two exactly similar cells were used, one for the solution under investigation and one for the solvent. The thickness of the liquid layer was exactly 20 mm. Measurements were carried out at room temperature.

The blood-serum extract investigated was the ether-alcohol extract referred to above in Part I. The concentration of this solution, as first extracted, naturally varied from case to case. In order therefore to facilitate the absorption determinations and to ensure more easily comparable results, the concentration was always adjusted by trial (either by adding solvent or by carefully evaporating down) until the observed absorption lay in the range most suitable for measurement. After the determinations the extract was returned to H. H. Barber for the chemical examination described in Part I.

The quantity actually measured was the ratio of the intensity I_0 of the beam incident upon the solution under investigation to the intensity I of the transmitted beam. The simultaneous use of a comparison vessel containing pure solvent introduced full correction for reflection and for absorption by the vessel and solvent. The results are expressed as extinction coefficients ϵ, defined by the equation

$$\epsilon = \frac{1}{d} \log_{10} \frac{I_0}{I},$$

in which d is the thickness of the liquid layer in cm. In our case $d = 2{\cdot}000$. It is to be noted that the concentration does not appear in this equation. Assuming the validity of Beer's Law, the quantity ϵ would have to be divided in each case by the molecular concentration of the solution in order to obtain the strictly comparable quantity, the molecular extinction coefficient. Actually no account is taken of the small differences in the concentrations of the extracts studied. This means that the whole set of ϵ-values for any particular extract is multiplied by a certain small concentration factor, as compared with the set of values for another extract. Since, however, we are not concerned with absolute values, but only with the relative shapes of the respective absorption curves, this circumstance is of no importance. It should be mentioned also that the extinction coefficient ϵ used here differs from the quantity α used by the previous workers in that the equation defining ϵ contains a logarithm to the base 10, whereas the exactly analogous equation defining α contains a logarithm to the base e. This introduces a constant conversion factor which, however, is again of no significance since we are concerned with relative, and not with absolute, values.

The results of the determinations for 20 cases are given in Table II. The first column gives the reference number of the case and the second the clinical diagnosis, C denoting cancer and N non-cancer. For further details of the cases see Table I in Part I, in which the same reference numbers are used. The rest of Table II contains the observed values of ϵ in columns headed by the respective wave-lengths expressed in mμ.

Table II.

Ref. no.	Clinical diagnosis	Observed values 313·0	302·3	296·8	289·4	280·2	275·6	265·3	253·7	248·1	240·0
35	C	0·158	0·215	0·249	0·310	0·390	0·435	0·518	0·599	0·668	—
36	N	0·213	0·248	0·266	0·330	0·386	0·414	0·468	0·574	0·639	0·754
37	N	0·161	0·205	0·246	0·287	0·344	0·376	0·461	0·663	0·762	0·91
38	C	0·179	0·291	0·360	0·468	0·578	0·644	0·785	0·96	1·01	—
39	N	0·154	0·227	0·281	0·373	0·508	0·595	0·800	1·08	1·17	—
40	N	0·132	0·207	0·269	0·383	0·545	0·648	0·889	1·15	—	—
51	C	0·106	0·155	0·190	0·256	0·360	0·421	0·545	0·749	0·894	—
52	C	0·107	0·165	0·201	0·260	0·343	0·396	0·546	0·813	0·97	—
53	N	0·093	0·158	0·205	0·291	0·422	0·513	0·726	1·09	—	—
54	N	0·100	0·132	0·160	0·207	0·265	0·306	0·382	0·560	0·690	0·892
55	N	0·096	0·139	0·169	0·221	0·310	0·368	0·504	0·747	0·91	—
56	C	0·092	0·137	0·166	0·219	0·295	0·348	0·483	0·726	0·854	—
57	N	0·094	0·140	0·176	0·241	0·339	0·403	0·553	0·818	0·974	—
58	C	0·089	0·128	0·158	0·207	0·276	0·323	0·416	0·584	0·706	—
59	C	0·097	0·122	0·142	0·179	0·226	0·245	0·276	0·310	0·354	0·456
60	N	0·085	0·110	0·130	0·162	0·203	0·225	0·257	0·324	0·387	0·554
61	C	0·101	0·137	0·161	0·201	0·259	0·282	0·313	0·382	0·466	0·703
62	N	0·092	0·135	0·160	0·196	0·239	0·271	0·332	0·448	0·487	0·579
63	N	0·092	0·122	0·145	0·178	0·227	0·250	0·302	0·391	0·465	0·609
64	C	0·118	0·167	0·202	0·261	0·348	0·393	0·503	0·683	0·810	1·08

The procedure in carrying out the determinations was to start with the longest wave-length and work through progressively to the shortest. In a large number of cases, however, values measured at the beginning of a run were redetermined afterwards. The agreement obtained was always within the limits of experimental error given above.

Discussion of results

Dannmeyer *et al.* [1931] express their results in the form of absorption curves in which the measured absorption coefficients α are plotted against wave-lengths. They reproduce 10 of these curves, 5 for cases of cancer and 5 for non-cancer, the examples being chosen as being "especially characteristic" (p. 652). They claim that the form of the curve in any given case furnishes a diagnostic test for the presence or absence of cancer. The test is actually effected either (*a*) by purely visual inspection of the curve or (*b*) by a formal method of analysis. As far as (*a*) is concerned, it is stated to be "clearly apparent" from the 10 examples reproduced that the curves of the cancer show "an essentially more uniform course" (*einen wesentlich einheitlicheren Verlauf*, p. 652) than those of the non-cancer cases. The present writer is unable, however, to perceive this qualitative difference by visual inspection of the curves in question. As the test appears to be so indefinite for examples chosen as especially characteristic, it is presumably even more so in average cases.

In the hope that the characteristic difference between cancer and non-cancer absorption curves, if it exists, might be more apparent from the results of the present investigation, these results were plotted in the same manner; but visual inspection of the curves obtained reveals no characteristic differences such as might serve as basis for a diagnostic test for cancer.

It may be noted that for all our curves of ϵ against λ the slope is everywhere negative, whereas some of the curves reproduced by the previous workers show regions of positive slope in the same wave-length range.

Reproduction of our curves, which are plotted from the data given in Table II, is prohibited by considerations of space.

Though purely visual inspection is claimed by the previous workers to suffice for purposes of diagnosis, they also give an account of a formal method (*b*) whereby the absorption curve under consideration is analysed into a so-called "basic curve" (*Grundkurve*) and a number of superimposed "partial bands" (*Teilbanden*). The latter are claimed to be characteristically different in cancer cases, as compared with non-cancer cases. For the purpose of the analysis it is found expedient, instead of plotting the absorption coefficient α against the wave-length λ, to plot log α against the reciprocal of λ^2. The "basic curve" then becomes a straight line. From the four examples given there appears, however, to be a certain arbitrariness involved in deciding the exact position of this line. The "basic curve" having been drawn, its ordinates are subtracted from those of the original curve and the differences replotted as ordinates of a new curve which we may call the selective absorption curve. This is finally analysed into a number (as many as six in one of the examples) of superimposed "partial bands", the sum of whose ordinates at any value of λ is equal to the ordinate of the curve. Now it is clear that small experimental errors in the determination of the original total absorption curve become relatively much greater for the ordinate differences which are plotted to obtain the selective absorption curve, and also that the values of these differences are affected seriously by a small uncertainty in the position of the "basic curve". The number of known points on the selective absorption curve is limited, being equal to the number of wave-lengths at which the original absorption determinations were made, and the course of the curve is in general very irregular. Hence it appears that a certain degree of arbitrariness is necessarily associated with an analysis into a number of overlapping "partial bands", even though these bands are required to satisfy certain conditions.

These considerations would seem to detract from the applicability of this method of analysis to the absorption curve of such a complicated system as the serum extract investigated. Since, therefore, the object of the present investigation was to find out whether ultraviolet absorption measurements on the extract could furnish a diagnostic test for cancer, it was thought best (in the absence of a thoroughly unambiguous method of analysis) to confine attention to purely visual inspection of the form of the absorption curves obtained. As stated above, our results on 20 cases give no evidence of the possibility of a diagnostic test of the kind sought.

SUMMARY.

Part I.

From the results of the examination of 41 specimens of blood serum obtained from both normal and cancer cases, there does not appear to be any significant difference in the saponification values of the serum lipoids as isolated under the conditions laid down in the paper of von Noël [1931].

The "Krebszahlen" calculated in the prescribed manner do not show differences sufficiently characteristic to be of diagnostic value.

Part II.

The investigations of this part constitute an independent test of the claim of previous workers to have obtained a method of diagnosing cancer by the form of the ultraviolet absorption curve of an extract from blood serum. An account is given of ultraviolet absorption measurements made with a double quartz

monochromator and photoelectric spectrophotometer. Results are tabulated for 20 cases, 9 of which were clinically diagnosed as cancer and 11 as non-cancer. These results furnish no evidence for the possibility of the diagnostic method under test.

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CCVII. SOME OBSERVATIONS ON THE REDUCTION OF IRON BY TISSUE EXTRACTS AND BY ASCORBIC ACID, WITH A NOTE ON THE STABILIZATION OF ASCORBIC ACID SOLUTIONS.

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The investigations of a large number of workers have made possible a general conception of the metabolism of iron. The retention of iron in the body, blood formation and other aspects of iron metabolism are conditioned by the reduction of food iron in the alimentary tract and its absorption from the duodenum in the ferrous state [McGowan, 1930; Lintzel, 1933]. The absorbed iron is believed to be oxidized in the blood and transported, presumably as a ferric compound of globulin [Starkenstein & Harvalik, 1933], to the liver where it is either utilized in haemoglobin synthesis or stored largely in the reduced state. The presence of ascorbic acid, in relatively large concentration in the intestinal mucosa [Hopkins, 1934; Zilva, 1935] and in the liver, invites speculation as to the role which this substance may play in the absorption of iron from the alimentary tract and in the reduction of blood iron in the liver.

The secondary anaemia of human and guinea-pig scurvy is well known. How far the anaemia is the result of a derangement of iron metabolism due to the lack of a specific reducing substance (ascorbic acid) is not definitely known. Stacy & Chew [1932] believe the fundamental cause to be retarded erythropoiesis. The oral administration of iron was shown to be ineffective in alleviating the anaemia of human scurvy [Mettier *et al.* 1930]. In a series of experiments with guinea-pigs in which scurvy was produced by feeding the diet of Harris *et al.* [1932], we observed an anaemia of similar severity to that found by Stacy & Chew [1932]. During the recovery which followed the injection of 3 mg. of ascorbic acid daily the haemoglobin and red-cell count returned to the normal level. When next the ascorbic acid was withheld and each guinea-pig received instead a daily intraperitoneal injection of 3·2 mg. of Fe'' or Fe''' in the form of Mohr's salt or ferric chloride the animals again lost weight, all became anaemic and died of scurvy within 12 days. When 6 guinea-pigs were fed the same diet and received daily injections of 3·2 mg. Fe'' as Mohr's salt, severe scurvy marked by inanition, rapid loss in weight and the development of a pronounced secondary anaemia developed in all the animals. We believe with Stacy and Chew that the hemorrhages which occurred in any of these animals could not be responsible for the degree of anaemia which ensued.[1]

The failure to maintain normal haematopoiesis in animals suffering from advanced scurvy by the parenteral administration of iron is not surprising. The animals received a diet which, owing to the inclusion of egg-yolk, had a high iron content (0·021 % Fe). Presumably the iron stored in the liver should have been sufficient to permit the haemoglobin content of the blood to be maintained at the normal level during the short period of extreme inanition. It may be concluded that the anaemia of scurvy is not due to a diminished absorption of iron. Eichholtz & Unrath [1935] have found that feeding mice with ascorbic acid along with iron did not increase the amount of so-called "catalytically active" iron available to the organism.

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[1] In view of the negative findings in these experiments it was considered that no useful purpose would be served by giving the detailed results.

If a derangement in iron metabolism is responsible for the anaemia of scurvy it would appear to be localized in the mechanism which brings about the reduction of iron in the liver. The results of the experiments just described would not exclude this possibility if, as Starkenstein & Harvalik [1933] believe, injected ferrous iron is rapidly oxidized by the blood. In fact direct evidence in this regard is not easily obtained. The experiments *in vitro* now to be described are mainly concerned with certain aspects of the reduction of iron salts by tissue extracts and by ascorbic acid and constitute an attempt to obtain indirect evidence linking ascorbic acid in the chain of processes involved in the metabolism of iron.

1. THE REDUCTION OF FERRIC ION BY TISSUE EXTRACTS.

The technique and the accuracy of the method employed to determine the amount of iron reduced under the conditions of succeeding experiments is illustrated below in a brief summary of the results of an experiment which show, as anticipated, that the reaction between ascorbic acid and ferric ion involves a two-electron change.

Composition of the test solution:

8 ml. of phthalate or acetate buffer.
1 ml. of a ferric chloride solution containing 0·50 mg. Fe.
1 ml. of approximately 0·001 *M* ascorbic acid solution.
4·4 mg. B.D.H. ascorbic acid plus 3·0 ml. 0·001 *M* solution of sodium diethyldithiocarbamate and diluted to 25 ml. with ordinary distilled water.

This solution was found, by iodine titration, to contain 0·173 mg. ascorbic acid per ml. A note at the end of this paper explains the use of the carbamate reagent.

The final *p*H of the mixture was determined by the quinhydrone electrode. Solutions of *p*H 2·2 to 5·5 were employed.

Titanium titration:

Using the micro-technique of McFarlane [1936] repeated titrations of 2·0 ml. aliquots of the above mixtures gave values varying from 0·190 to 0·199 ml. of titanous chloride solution. The total iron (0·10 mg. Fe''') in a 2 ml. aliquot required 0·249 ml. of the titanous chloride solution for its quantitative reduction.

Therefore 0·190 ml. $TiCl_3$ solution is equivalent to 0·0763 mg. Fe and 0·199 ml. $TiCl_3$ solution is equivalent to 0·080 mg. Fe.

1 ml. 0·001 *M* ascorbic acid solution at *p*H 2·2–5·5 reduced 0·120–0·102 mg. Fe.

Calculated on the basis that one molecule of ascorbic acid reduces two atoms of iron.

1 ml. 0·001 *M* ascorbic acid solution should reduce 0·112 mg. Fe.

At physiological hydrogen ion concentrations ascorbic acid, according to Szent-Györgyi [1934] forms a stable complex with ferrous iron. A simple calculation, using the values for the "inorganic" iron content of tissues as given by Tompsett [1935] or McFarlane [1934] shows that the amount of ascorbic acid usually considered to be contained in these tissues is more than sufficient to hold the iron in the reduced state. Measurement of the degree to which trichloroacetic acid extracts of tissues reduce 2:6-dichlorophenolindophenol forms the basis of the well known procedure of Birch *et al.* [1933] for the estimation of ascorbic acid. It becomes of interest to determine the degree to which tissue extracts will reduce ferric ion relatively to their reducing intensity as measured by the dye titration. These measurements have been made and the results are presented in Table I.

The general procedure employed to obtain these data was to triturate a weighed quantity of the tissue with 5 ml. of a 20 % solution of trichloroacetic acid and a small amount of quartz sand.[1] The suspension was centrifuged and the precipitate washed with 20 ml. of 1 % trichloroacetic acid solution. The combined centrifugates were made up to a definite volume. An aliquot was transferred to a small Erlenmeyer flask and to it was added an equal volume of phthalate-HCl buffer of *p*H 4·5 and a measured volume, usually 2 ml., of a standard ferric chloride solution containing 0·05 mg. Fe''' per ml. The *p*H of the mixture was approximately 2·5. The amount of iron reduced was determined and its equivalent as ascorbic acid calculated by the procedure already described.

According to Fugita & Iwataki [1935] the estimation of ascorbic acid is more accurately carried out using metaphosphoric acid instead of trichloroacetic acid extracts. Simultaneous determinations have also been made using their procedure.

Table I. *The ascorbic acid content of various tissues as calculated from the amount of iron reduced by trichloroacetic acid extracts and from the amount of dye reduced by trichloroacetic acid or metaphosphoric acid extracts.*

	Trichloroacetic acid extract		Metaphosphoric acid extract	% of indophenol value estimated by	
Tissue	Iron reduction	Dye titration	Dye titration	(*a*) Iron reduction	(*b*) Colorimetric‡
1. Rat livers	0·21*	0·29	0·27	72	67
	0·22	0·28	—	75	—
2. Rat spleens	0·30	0·50	—	60	100
	0·31	0·48	—	64	88
3. Rat kidneys	0·14	0·34	0·22	41	46
	0·13	0·35	—	40	62
4. Chick livers	0·13	0·38	0·35	—	—
	0·11	0·37	0·37	—	—
5. Ox adrenals:					
A. Fresh	0·59	1·19	1·21	53	—
	0·78	1·24	1·30	63	—
	0·58	1·67	—	34	30§
	0·49	1·62	—	30	—
B. Frozen, 4 months	0·44	0·96	0·96	46	—
	0·42	0·96	—	44	—
6. Orange juice	0·45†	0·47	—	96	—

* mg. per g. of tissue—average value of duplicate analysis.
† mg. per ml.
‡ Taken from the paper of Fugita *et al.* [1935, Table VIII].
§ Guinea-pig adrenals.

It will be observed (Table I) that the amount of ascorbic acid in these animal tissues as calculated from the amount of iron reduced is always lower than that obtained by the dye titration. This is particularly so in the case of the kidney and the adrenal. In general the results by iron reduction are in good agreement with those which were obtained by Fugita *et al.* [1935] using a colorimetric procedure involving the reduction of sodium tungstate in alkaline solution. According to Van Eekelen [1935] this colorimetric method gives low results when applied to the adrenal gland owing to the interfering action of adrenaline. Difficulties attending the estimation of ascorbic acid in kidney extracts are mentioned by Hopkins & Slater [1935] who found that the results are dependent upon the rate at which the dye titration is carried out. It still remains to be determined whether any of the values given in Table I represent the true ascorbic acid content of the particular tissue.

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[1] The quartz sand was freed from iron by extraction with hot concentrated HCl.

Ascorbic acid estimations in orange juice by iron reduction and by dye titration have consistently given results which were practically identical.

Millikan [1935] has shown that the rate of reduction of 2:6-dichlorophenolindophenol by cysteine is very much slower than that obtained with ascorbic acid. We have observed a similar difference in the rates at which ascorbic acid and glutathione reduce ferric ion. When 1 ml. of a 0·001 M solution of ascorbic acid is added to each one of a series of solutions ranging in pH from 3·1 to 5·0 and composed of 3·0 ml. phthalate-HCl or NaOH buffer, 0·5 ml. ferric chloride solution containing 0·05 mg. Fe''' and 0·5 ml. of 0·001 M $\alpha\alpha'$-dipyridyl solution, the pink colour of ferrous dipyridyl develops immediately and in a few minutes reaches an intensity indicating quantitative reduction. When the experiment is repeated using 1 ml. of a 0·001 M solution of crystalline reduced glutathione instead of ascorbic acid no evidence of reduction is obtained in the first hour. A trace of colour is observed after 3 hours which develops slowly to reach maximum intensity in about 24 hours. It would thus appear that sulphydryl compounds exert little, if any, influence upon the reduction of iron by tissue extracts under the conditions of these experiments.

2. THE REDUCTION OF IRON SALTS BY ASCORBIC ACID.

Assuming that ascorbic acid plays some role in the reduction of the iron in tissues, the question now arises as to the nature of the iron compound(s) reduced at the hydrogen ion concentration of the tissue. Obviously some complex of iron and not ferric ion is involved. When ferric chloride is added to a series of Sørensen's phosphate mixtures (pH 4·7–8·0) the iron is precipitated at approximately pH $>$6·0 and the precipitated iron, we have found, is not reduced by ascorbic acid. We have already shown [McFarlane, 1934] that about 60 % of the non-haematin iron in liver tissue is precipitated in the trichloroacetic acid fraction and is reducible by sodium hydrosulphite. Further, when a solution of ferric chloride is added to liver pulp the added iron is completely precipitated by 50 % alcohol. The iron extracted from tissues by trichloroacetic acid is all in the ferrous form and presumably is present in the tissues as a soluble ferrous complex possibly as ferrous ascorbate. Considering these facts and the observations of Starkenstein and Harvalik, already referred to, it would appear probable that an iron-protein complex is involved.

We have taken lecithovitellin as an example of such an iron-protein compound. To a solution of lecithovitellin, prepared according to McFarlane [1932], and buffered at pH 7·4 were added $\alpha\alpha'$-dipyridyl and ascorbic acid. A pink colour developed slowly and reached an intensity indicating quantitative reduction of the iron in a few hours. Reduced glutathione under the same conditions also reduced the iron of lecithovitellin but at an even slower rate.

It is now well known that hydroxy-organic acids combine with iron to form soluble unionized compounds. The influence of pH on the reduction of ferric lactate by ascorbic acid was next investigated.

At pH $<4 \cdot 1$ one mole of ascorbic acid reduced 2 atoms Fe.
At pH 4·6–5·2 one mole of ascorbic acid reduced 1 atom Fe.
At pH $>5 \cdot 6$ ascorbic acid failed to reduce any iron.

According to Smythe [1931] lactic acid behaves as a dibasic acid when titrated in the presence of ferric chloride, the pK_a for the alcoholic hydroxyl group being about 3·8. With ferric glutamate solutions we found that:

At pH $<4 \cdot 9$ one mole of ascorbic acid reduced 2 atoms Fe.
At pH 5·1–5·9 one mole of ascorbic acid reduced 1 atom Fe.
At pH $>6 \cdot 1$ no reduction took place.

Smythe & Schmidt [1930] have observed that glutamic acid will retain some iron in solution at pH 5·0 but that it is all precipitated at pH 6·0.

The inference from these findings is that some form of iron-protein combination is a conditioning factor in so far as the reduction of tissue iron by ascorbic acid is concerned.

3. A NOTE IN REGARD TO THE INHIBITION OF THE CATALYSIS OF THE OXIDATION OF ASCORBIC ACID BY HEAVY METALS.

Mawson [1935] has shown that whilst the action of copper and of ferrous or ferric iron in catalysing the aerobic oxidation of ascorbic acid is retarded by glutathione, cysteine, cystine and H_2S the same protective action of tissue extracts is not quantitatively accounted for on the basis of their content of sulphydryl compounds. Recently Hunter [1935] has isolated taurine in considerable amounts from the adrenal gland. Taurine, we have found, exerts no influence upon the copper catalysis of ascorbic acid oxidation. At the same time we have made some general observations in regard to the stabilizing action of several sulphur-containing compounds.

We first found that a concentration of copper between 1×10^{-4} and 5×10^{-5} mg. just effects the complete oxidation, in 1 hour at 38°, of 0·18 mg. of ascorbic acid in a total volume of 5 ml. of phosphate solution,[1] pH 6·6. The effects of several

Table II. *Showing the influence of various sulphur compounds on the catalysis of ascorbic acid oxidation by copper.*

Composition of the test solutions:

1·0 ml. 0·001 *M* ascorbic acid solution (standardized by iodine titration).
1·0 ml. copper sulphate solution (2×10^{-4} mg. Cu per ml.).
1·0 ml. 0·0001 *M* solution of the sulphur compound.
2·0 ml. phosphate buffer pH 6·6.

Sulphur compound	% oxidation after incubation at 38° for 1 hour
Control—no sulphur compound	100
Taurine	100
Glutathione	85
Cysteine hydrochloride	33
Cystine	27
Sodium diethyldithiocarbamate	zero

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[1] Sørensen's phosphate mixture. The primary and secondary phosphates employed were three times recrystallized from glass-distilled water. All filtrations were made using Jena sintered filters.

compounds in inhibiting this oxidation were next investigated with the results shown in Table II. The general procedure used to estimate the reduced ascorbic acid remaining in the test solution was to add an excess of a solution of 2:6-dibromophenolindophenol, which had been standardized against a standard ascorbic acid solution and against titanous chloride, and to back-titrate the excess of the dye with titanous chloride.

It is well known that at extreme dilutions sodium diethyldithiocarbamate forms an orange-yellow coloured undissociated complex with copper. Compared with the other sulphydryl compounds the action of glutathione was slight. Taurine or glycine even in 0·01 *M* solution has no effect. Even in the presence of the carbamate reagent ascorbic acid solutions in ordinary distilled water become increasingly unstable at *p*H $>7{\cdot}5$.

Further experiments (Table III) have shown that the oxidation of the ascorbic acid in orange juice left standing in the laboratory for 9 hours is almost completely inhibited by adding sodium diethyldithiocarbamate and αα′-dipyridyl but is not affected by adding either reagent singly. In fact the addition of αα′-dipyridyl alone appeared to result in an acceleration of the oxidation. Glutathione has practically the same effect as the combination of dipyridyl and carbamate reagent.

Table III. *Showing the effect of αα′-dipyridyl, sodium diethyldithiocarbamate or glutathione on the rate of oxidation of ascorbic acid in orange juice.*

	Ascorbic acid (mg./ml. orange juice)		
Test solutions	Zero hour	After 9 hours	% oxidation
1 ml. orange juice + 2 ml. H_2O*	0·46	0·18	61
1 ml. orange juice + 1 ml. 0·001 *M* dipyridyl + 1 ml. H_2O	0·41	0·09	78
1 ml. orange juice + 2 ml. 0·001 *M* dipyridyl + 1 ml. H_2O	0·39	0·06	85
1 ml. orange juice + 1 ml. 0·001 *M* carbamate + 1 ml. H_2O	0·44	0·16	64
1 ml. orange juice + 1 ml. 0·001 *M* carbamate + 1 ml. 0·0001 *M* dipyridyl	0·46	0·42	9
1 ml. orange juice + 2 ml. 0·02 *M* glutathione	0·46	0·40	13

* Three times redistilled from glass and also used as the solvent for the other reagents employed.

The observation of Mawson in regard to the factors influencing the stability of ascorbic acid in fruit juice is therefore confirmed and extended. The applications of these reagents in facilitating the ascorbic acid analysis of plant juices and in the preparation of stable aqueous solutions of ascorbic acid are obvious.

SUMMARY.

The results of some experiments *in vitro* on the reduction at different *p*H values of ionic iron, iron in combination with lactic acid, glutamic acid or protein by ascorbic acid indicate that the reduction of tissue iron *in vivo* by ascorbic acid must involve some form of iron-protein complex. The relative capacities of extracts of several tissues to reduce Fe''' and 2:6-dichlorophenolindophenol have been quantitatively measured.

The catalysis of ascorbic acid oxidation by copper is inhibited by the following substances, in order of decreasing activity, sodium diethyldithiocarbamate, cystine, cysteine and glutathione but not by taurine or glycine. The aerobic oxidation of ascorbic acid in orange juice is inhibited by adding αα′-

dipyridyl and sodium diethyldithiocarbamate together. It is not affected by adding the carbamate reagent alone but may actually be accelerated by the single addition of dipyridyl.

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CCVIII. CARBOHYDRATE CATABOLISM IN CEREBRAL CORTEX.

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Pasteur [1861; 1875] suggested that the presence of oxygen decreases the rate of sugar destruction by yeast, and also suppresses or diminishes the accumulation of anaerobic cleavage products. Accordingly the Pasteur effect may be defined as the effect of oxygen in reducing carbohydrate catabolism and in diminishing or suppressing the accumulation of the products of anaerobic metabolism. This definition involves two characteristics. It is evident that the first characteristic of oxygen in causing the Pasteur effect of necessity involves the second, since if there is decreased catabolism of carbohydrate less cleavage products must be formed. However, decrease in the formation of anaerobic cleavage products does not necessitate decreased carbohydrate catabolism. It is thus merely redundant to add the second characteristic in defining the Pasteur effect, although in the loose definition often given, it is often this characteristic which is mainly accentuated.

Accordingly it is necessary to demonstrate that the presence of oxygen causes a decrease in the rate of carbohydrate catabolism for it to be certain that the Pasteur effect is in operation. This has been shown by Meyerhof [1920] in the case of the destruction of glycogen by frog muscle and by Negelein [1925, 1] in the case of the red blood corpuscles of the goose and the rabbit acting on glucose. Negelein, however, measured the rate of carbohydrate destruction in the presence of cyanide instead of the anaerobic rate.[1] In no other tissue has the Pasteur effect been unequivocally demonstrated. Most work on the subject involves measurements of oxygen uptake as well as of glycolysis.[2] From the values of the respiration and the glycolysis under aerobic conditions and of the anaerobic glycolysis it may be inferred that the carbohydrate destruction is reduced in the presence of air, although this inference does not amount to a rigid proof.

The present communication shows how the rates of anaerobic and aerobic carbohydrate catabolism by brain cortex slices can be calculated indirectly from the rates of respiration and glycolysis and further demonstrates that this method of calculation is justified by actual measurements of the rates of sugar disappearance under these various conditions. It is thus shown definitely that the Pasteur effect is in operation in slices of cerebral cortex acting on glucose *in vitro*.

Experimental results and discussion.

In considering this problem we adopt certain new symbols to express the rate of carbohydrate disappearance. The rate of disappearance of hexose is expressed by the symbol Q_{C_6}; this represents the number of μl. of hexose sugar expressed as a gas at N.T.P., which disappears per mg. dry weight of tissue per hour. Since it is conventional to represent the rate of disappearance of a substance by a negative number, Q_{C_6} is always negative if sugar is being destroyed. The symbol $Q_{C_6}^{N_2}$ represents the rate of sugar disappearance under anaerobic conditions, whilst $Q_{C_6}^{O_2}$ is the aerobic rate of disappearance. When $Q_{C_6}^{O_2}$ is numerically less than $Q_{C_6}^{N_2}$ the Pasteur effect is in operation, since the rate of carbohydrate destruction is less in oxygen than in anaerobiosis.

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[1] Except in the case of two experiments which he performed on tumour tissue and in which the effect of oxygen on the rate of sugar destruction was to cause only a small percentage diminution.

[2] By the term glycolysis we mean either the formation of lactic acid in animal tissues or of ethyl alcohol and carbon dioxide in plants.

In cerebral cortex it is possible to calculate Q_{C_6} from the rates of glycolysis and respiration.

The equations

$$C_6H_{12}O_6 = \underset{\text{Lactic acid}}{2C_3H_6O_3} \qquad \text{......(1)},$$

and

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O \qquad \text{......(2)},$$

represent respectively the glycolytic and respiratory processes occurring in brain supplied with glucose. The respiration of other intracellular substrates apparently does not occur in the presence of added glucose or lactate, since with these substances the respiration remains constant, whilst without substrate it falls rapidly [*v.* Dixon, 1935].

Further by equation (1)

$$Q_{C_6}^{N_2} = -\frac{Q_M^{N_2}}{2}*,$$

and by equations (1) and (2)

$$Q_{C_6}^{O_2} = -\frac{Q_M^{O_2}}{2}* + \frac{Q_{O_2}}{6}*.$$

Q_{C_6} is naturally negative as it expresses a rate of disappearance; this explains the negative signs preceding Q_M in the above equations, since Q_M is positive when lactic acid is being formed. Q_{O_2} is always negative.

We will now consider an example of the calculation of $Q_{C_6}^{N_2}$ and $Q_{C_6}^{O_2}$. In an experiment on slices of rabbit's brain cortex, using the two-vessel method of Warburg [1924], I found the following metabolic rates (see Table I):

Table I.

Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
−8·3	2·8	20·7

From these figures $Q_{C_6}^{N_2} = -\frac{20{\cdot}7}{2} = -10{\cdot}35$,

while

$$Q_{C_6}^{O_2} = -\frac{2{\cdot}8}{2} + \frac{-8{\cdot}3}{6} = -2{\cdot}78.$$

Thus $Q_{C_6}^{O_2}$ is numerically less than $Q_{C_6}^{N_2}$. We therefore conclude that the Pasteur effect is operating. It might, however, be argued, although the sugar disappearance, as calculated above, is reduced by the presence of oxygen, that the absolute rate of disappearance is not really reduced, but that some intermediary other than lactic acid is accumulating. This was rendered unlikely by the results of some experiments kindly communicated to me by Dr E. G. Holmes.

Dr Holmes measured the aerobic rate of sugar destruction by brain cortex slices and obtained simultaneous measurements of the lactic acid production (by chemical method) and of the CO_2 of respiration (by the method of M. Dixon & Keilin). It is clear from his results that the sugar destroyed can be mainly accounted for by lactic acid production and respiration and that at any rate there is no accumulation of an intermediary (other than lactic acid) in amount sufficient to account for the effect of oxygen in reducing glycolysis.

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* These symbols are defined by Warburg [1925] and Negelein [1925, 2].

To justify the above conclusions it was, however, necessary to measure simultaneously the rates of sugar disappearance in oxygen and in nitrogen. Accordingly experiments were performed in which definite amounts of glucose were added to brain slices suspended in bicarbonate Ringer. The slices were then incubated both under aerobic and anaerobic conditions, and the sugar remaining in the Ringer was estimated by the Hagedorn-Jensen method.

In actual practice 0·2 ml. of 0·3–0·4 % glucose was added to the brain slices (3–10 mg. dry weight) suspended in Ringer. At the end of the experiment 1 ml. of 40 % trichloroacetic acid was added to the suspension. After standing 15 min. the suspension was filtered quantitatively into a 25 ml. graduated flask and the sugar content estimated. The slices remaining on the filter paper were dried and weighed. The original sugar present was estimated in the presence of identical concentrations of trichloroacetic (and Ringer's solution). In one set of experiments the original sugar was incubated and filtered in the same manner as the solutions containing the slices. This did not affect the final result. The initial concentration of sugar in the Ringer was 0·04–0·05 %, the total amount of sugar added being 0·5–0·8 mg. The experiments lasted for 1 hour. The results of these experiments are tabulated below (Table II):

Table II.

	mg. sugar loss per hour	Dry wt. of slices in mg.	Sugar loss in mg. per mg. dry wt. per hour	Q_{C_6}
Exp. 1.				
A slices incubated in O_2 containing 5 % CO_2	0·147	7·70	0·019	2·4
B slices incubated in O_2 containing 5 % CO_2	0·133	6·24	0·021	2·6
C slices incubated in N_2 containing 5 % CO_2	0·287	4·64	0·062	7·7
D slices incubated in N_2 containing 5 % CO_2	0·247	5·05	0·049	6·1
Exp. 2.				
A slices incubated in O_2 containing 5 % CO_2	0·161	5·81	0·028	3·5
B slices incubated in O_2 containing 5 % CO_2	0·209	8·04	0·026	3·2
C slices incubated in N_2 containing 5 % CO_2	0·384	5·51	0·070	8·7
D slices incubated in N_2 containing 5 % CO_2	0·350	5·37	0·065	8·1
Exp. 3.				
A slices incubated in O_2 containing 5 % CO_2	0·159	5·04	0·032	– 4·0
B slices incubated in O_2 containing 5 % CO_2 (0·1 *M* KCl added to Ringer)	0·318	3·54	0·090	– 11·2
C slices incubated in N_2 containing 5 % CO_2	0·292	3·48	0·084	– 10·5

(In all the experiments the vessels containing the slices were shaken in a bath at 37°.)

It is clear from the above results that $Q_{C_6}^{O_2}$ is always numerically less than $Q_{C_6}^{N_2}$. In other words the Pasteur effect is operating in cerebral cortex. Further, the addition of *M*/10 KCl inhibits the Pasteur effect and raises the aerobic catabolism of carbohydrate approximately to the normal anaerobic level [*v.* Ashford and Dixon, 1935]. We thus find that the indirect method of demonstrating the Pasteur effect is, in brain at any rate, substantiated by absolute measurements of the rate of sugar disappearance.

SUMMARY.

1. The data necessary for the demonstration of the Pasteur effect are defined.

2. Symbols representing the rates of carbohydrate destruction under aerobic and anaerobic conditions are introduced—the symbols are $Q_{C_6}^{O_2}$ and $Q_{C_6}^{N_2}$ respec-

tively. The calculation of these rates from the rates of respiration and glycolysis is described. If $Q_{C_6}^{O_2}$ is numerically less than $Q_{C_6}^{N_2}$ the Pasteur effect is in operation. This is shown to follow from the values of the respiration and glycolysis in cerebral cortex.

3. The absolute rates of glucose destruction by brain cortex slices under aerobic and anaerobic conditions have been measured. It is shown that $Q_{C_6}^{O_2}$ is numerically less than $Q_{C_6}^{N_2}$ when these quotients are measured directly as well as when they are calculated from the respiration and glycolysis.

4. The addition of potassium chloride (one of the so-called inhibitors of the Pasteur effect) raises the aerobic destruction of sugar to the anaerobic level.

I wish to thank Sir F. G. Hopkins for his kind interest in this work, and also Dr E. G. Holmes and Dr Malcolm Dixon for their advice and criticism.

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CCIX. THE EFFECT OF RISE IN TEMPERATURE ON THE CARBOHYDRATE CATABOLISM OF CEREBRAL CORTEX.

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The effect of rise in temperature on the respiration and glycolysis of animal tissues has been studied by Kubowitz [1929] and by Nakashima [1929]. Kubowitz using frog retina showed that aerobic glycolysis suddenly becomes apparent above 35°. The Pasteur effect is abolished above this temperature since the Meyerhof quotient descends to zero. Nakashima employed fish retina, which, like amphibian and unlike mammalian retina, shows little or no aerobic lactic acid production at normal temperatures. Nakashima found that in fish retina rise in temperature from 30 to 37·5° produces a large aerobic glycolysis, but here in contrast to frog's retina respiration is increased. The anaerobic glycolysis is also increased, but in spite of this Nakashima concluded that there is inhibition of the Pasteur effect embodying a fall in Meyerhof quotient at this high temperature. In the present communication the effects of abnormally high temperatures on the metabolism of cerebral cortex are described.

METHODS.

Slices of rabbit's brain cortex were employed. The respiration and glycolysis were measured by the two-vessel method of Warburg [1924]. Bicarbonate Ringer containing 0·2 % glucose was employed throughout.

The ordinary symbols denoting the rates of respiration and glycolysis are employed [*v.* Warburg, 1925; Negelein, 1925]. The symbols $Q_{C_6}^{O_2}$ and $Q_{C_6}^{N_2}$ as described by Dixon [1936] are also employed to represent the rates of sugar destruction in oxygen and in nitrogen respectively. These quotients are calculated from the respiratory and glycolytic rates as described by Dixon.

EXPERIMENTAL RESULTS AND DISCUSSION.

The results of a number of experiments at 37, 42 and 45° are shown in Table I.

Table I.

Temp. ... 37°					42°					45°				
Q_{O_2}	$Q_M^{O_2}$	$Q_{C_6}^{O_2}$	$Q_M^{N_2}$	$Q_{C_6}^{N_2}$	Q_{O_2}	$Q_M^{O_2}$	$Q_{C_6}^{O_2}$	$Q_M^{N_2}$	$Q_{C_6}^{N_2}$	Q_{O_2}	$Q_M^{O_2}$	$Q_{C_6}^{O_2}$	$Q_M^{N_2 *}$	$Q_{C_6}^{N_2}$
−6·8	1·6	−1·9	—	—	−8·0	2·3	−2·3	—	—	—	—	—	—	—
−8·7	3·4	−3·1	—	—	−8·2	4·1	−3·4	—	—	—	—	—	—	—
−8·1	3·0	−2·9	19·5	− 9·8	−9·4	4·4	−3·8	21·0	−10·5	—	—	—	—	—
—	—	—	21·0	−10·5	—	—	—	26·0	−13·0	−17·0	11·4	−8·5	39	−19·5
—	—	—	19·0	− 9·5	—	—	—	—	—	−13·0	8·0	−6·2	35	−17·5
			19·0	− 9·5									32	−16·0
−9·0	2·6	−2·8	—	—	—	—	—	—	—	−16·8	9·3	−7·4	—	—
—	—	—	—	—	—	—	—	—	—	−16·8	12·8	−9·0	—	—
										−16·7	8·0	−7·4		

* At 45° the anaerobic glycolysis was measured over the first period of 20 min., since it falls rapidly. The other value were obtained over periods of 40 min. or 1 hour.

At 42° there is only a small rise in the rate of aerobic and anaerobic carbohydrate catabolism above those at 37°. None of the metabolic reactions as measured by the various quotients appears much increased by this rise in temperature.

At 45°, however, the picture is entirely different. There is an enormous increase in the rates of respiration and anaerobic and aerobic glycolysis above the values at the lower temperatures. The rate of carbohydrate catabolism (Q_{C_6}) is accordingly also much increased at 45° above that at 37 and 42°.

It is clear that both the anaerobic and the aerobic rate of carbohydrate catabolism suffer an enormous increase on raising the temperature to 45°. The respiration is also increased. These effects are like those described in fish retina by Nakashima [1929]. However, it does not appear that the Meyerhof quotient is markedly reduced at 45°, values between 1·6 and 2·1 being obtained from the above figures. In normal brain the Meyerhof quotient is about 2. It would thus appear that the aerobic glycolysis is due to the fact that the respiration is no longer sufficient to suppress the increased glycolysis. Specific inhibition of the Pasteur effect has probably not occurred. The Pasteur effect is certainly working at 45° since oxygen still reduces the rate of carbohydrate catabolism at this temperature. It is possible, had Nakashima been able to calculate the Meyerhof quotient from the values of glycolysis and respiration obtaining initially, that in fish retina also the Meyerhof quotient would have proved not to be subnormal at high temperatures.

In measuring the anaerobic glycolysis at 45° it is necessary to obtain readings in the first 20 min. (i.e. after 20 min. equilibration time). The anaerobic glycolysis at 45° falls very rapidly. This fall is not experienced at 37 or 42°. This will be clear from Table II, which also shows that aerobic glycolysis and respiration at 45°, although changing with time, remain much more constant than does the anaerobic glycolysis.

Table II.

Successive 20 min. periods from commencement of readings	1st	2nd	3rd	4th	5th	6th	7th
Exp. 1.							
$Q_M^{N_2}$ at 37°	21	19	22	—	—	—	—
$Q_M^{N_2}$ at 42°	26	28	25	—	—	—	—
$Q_M^{N_2}$ at 45°	39	26	10	—	—	—	—
Exp. 2.							
$Q_M^{N_2}$ at 37°	19	17	16	17	19	17	—
$Q_M^{N_2}$ at 45°	35	22	17	11	6	6	4
$Q_M^{N_2}$ at 45°	32	18	11	7	—	—	4
$Q_M^{O_2}$ at 45°	8	8	13	10	12	6	5
Q_{O_2} at 45°	-13	-13	-15	-9	-9	-6	-5
Exp. 3.							
$Q_M^{N_2}$ at 45°	33	22	13	6	4	4	3
$Q_M^{N_2}$ at 45°	36	24	11	9	5	5	4
$Q_M^{O_2}$ at 45°	6	6	9	11	10	9	6
Q_{O_2} at 45°	-15	-12	-15	-11	-10	-9	-5
Exp. 4.							
$Q_M^{O_2}$ at 45°	9		15		13		—
Q_{O_2} at 45°	-17		-16		-13		—

The reversibility of this effect of high temperature in increasing the rate of carbohydrate catabolism has been studied. Two parallel experiments with tissue from the same brain were carried out at 45°. The manometers containing the slices were then transferred to a bath at 37°. Finally the manometers were reintroduced to the bath at 45°. The results are shown in Table III. In this table figures in the same vertical column represent the metabolic rates of the same tissue at the different temperatures.

Table III.

Temp. °C.	Exp. A			Exp. B		
	Q_{O_2}	$Q_M^{O_2}$	$Q_{C_6}^{O_2}$	Q_{O_2}	$Q_M^{O_2}$	$Q_{C_6}^{O_2}$
45	-16·8	12·2	-8·9	-16·7	8·6	-7·1
37	- 8·8	2·6	-2·8	- 7·7	3·0	-2·8
45	- 7·5	12·2	-7·4	- 7·4	11·5	-6·9

It appears that the effect on the aerobic glycolysis and carbohydrate catabolism is largely reversible. Respiration, aerobic glycolysis and carbohydrate catabolism ($Q_{C_6}^{O_2}$) all fall alike on transferring the tissue from the bath at 45° to that at 37°. The high glycolysis and carbohydrate catabolism are regained on reintroduction into the bath at 45°. It is not certain whether the effect on the respiration is reversible since the high rate cannot be recovered on reintroduction into the bath at 45°. The rate of respiration in any case falls at 45°. It is certain that the effect on the catabolism of carbohydrate is reversible. The low normal rate of destruction of carbohydrate is recovered at the lower temperature.

The main result of these experiments on the effect of temperature on the metabolism of brain is that a rise in temperature from 37 to 42° only produces a very slight increase in metabolic rate, whilst on raising the temperature to 45° the metabolism increases enormously. At 42° the respiration is scarcely raised above the normal value at 37°, nor is there any marked increase in aerobic glycolysis. At 45°, however, the respiration may be increased by 100% whilst marked aerobic glycolysis becomes evident, this being sometimes as much as six times as great as the normal value at 37°. Table IV shows the aerobic rates of carbohydrate (hexose) catabolism ($Q_{C_6}^{O_2}$) calculated from the rates of respiration and glycolysis at these three temperatures.

Table IV. $Q_{C_6}^{O_2}$ *in brain at various temperatures.*

37°	42°	45°
-2·9	-3·8	—
-3·0	-3·4	—
-1·9	-2·3	—
-2·8	—	-7·45
—	—	-8·9
—	—	-7·0

This table emphasises the sudden rise in the rate of aerobic carbohydrate catabolism seen between 42 and 45°. I have not yet obtained many figures for the rates of anaerobic glycolysis at these various temperatures, but from those described above it would seem that a similar, though not so marked, rise in the rate of carbohydrate catabolism occurs between 42 and 45°. Here the question is complicated by the fact that at 45° the rate of anaerobic glycolysis falls rapidly with time.

The relation between temperature and rate of chemical reaction is given by the equation of Arrhenius. This may be expressed in the form

$$\log \frac{k_1}{k_2} = K\,(1/T_1 - 1/T_2),$$

where k_1 and k_2 are the rates of reaction at the absolute temperatures T_1 and T_2 respectively, and K is a constant. From this it is seen that the logarithm of the velocity of the reaction is proportional to the reciprocal of the absolute temperature. There should in fact be a linear relation between the logarithm of the velocity and the reciprocal of the absolute temperature in the reactions of living cells.

The results so far obtained on the rates of carbohydrate catabolism at different temperatures are represented graphically in Fig. 1. The logarithm of the velocity of carbohydrate destruction ($\log_{10} - Q_{C_6}$) is plotted against the reciprocal of the absolute temperature. It is clear from Fig. 1 that the results do not subscribe to the Arrhenius law. The velocity of the reaction increases much more rapidly between 42 and 45° than one would expect from the rates at 37 and 45°. Crozier [1924; see also Barcroft, 1932], has emphasised the fact that many biochemical processes apparently "evade" the Arrhenius equation. In general the velocity at high temperatures is not so high as would be anticipated from those at lower temperatures if the Arrhenius law were followed. A good example of this is shown by the effect of temperature on the rate of reduction of methylene blue by various substances in the presence of *Bact. coli*, as shown by Cook [1930].

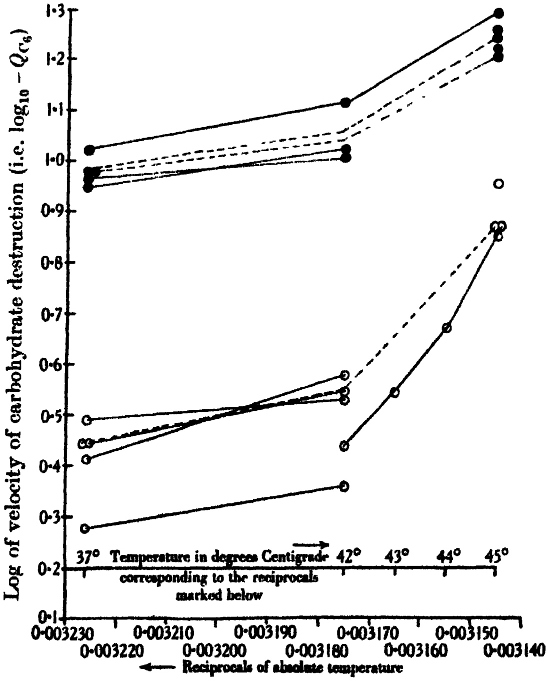

Fig. 1. The points connected by lines indicate observations obtained from the same brain. These lines are dotted where observations at the intermediate temperature (42°) were not obtained. • Signifies experiments conducted anaerobically. o Signifies experiments conducted aerobically.

Barcroft [1934] suggests that some governing mechanism is at work by which the cell resists the changes produced by a varying environment. In the case of cerebral cortex it would appear that this governing mechanism may break down above 42° and hence the abrupt rise in the rate of carbohydrate catabolism above this temperature.

These results are only of a preliminary nature and it is hoped later to extend the curves over a more complete range of temperature. One is, of course, only justified in regarding respiration and glycolysis as one reaction if both these processes have an initial common path, which is the reaction limiting them both. Otherwise we could not regard the total aerobic rate of carbohydrate destruction Q_{C_6} as measuring the rate of one reaction. But, at any rate, the same type of non-linear relation is obtained when the above treatment is applied to the respiration and glycolysis individually. The procedure followed above allows one to obtain readily a conception of the combined effect of respiration and glycolysis on the total rate of carbohydrate catabolism.

It is interesting to compare these effects of rise in temperature on the metabolism of rabbit's brain with the result of certain experiments of Marsh [1930]. Marsh noted that somewhere between 108 and 110° F. rabbits lose consciousness. 109° F. incidentally corresponds to 43° C. which is inside the temperature range (42–45°) where the metabolism of brain commences to become abnormal. It would seem that up to this level there is some regulating mechanism at work. When this breaks down carbohydrate catabolism runs riot in the brain cells and consciousness is simultaneously lost. It would thus appear that there may be a connection between the physiological process of consciousness and the rate of carbohydrate catabolism in the brain. Barcroft [1935] has pointed out the small size of the range of temperature over which the brain can function normally. It seems probable that the upper limit of this range is determined by this rapid rise in metabolic rate, though it also is possible that both the extent of this range and the metabolic rate are decided by some structural condition of the cell.

I have made one experiment so far in an attempt to ascertain more precisely the temperature at which this marked change in metabolism occurs. It consisted in measuring the metabolism of brain at intervals of 1° rise of temperature successively at the various temperatures between 42 and 45°. The results are seen in the following table (Table V):

Table V.

Temp. ° C.	Q_{O_2}	$Q_{M}^{O_2}$
42	- 8·4	2·7
43	- 10·8	3·4
44	- 11·3	5·5
45	- 11·0	11·3

(Bicarbonate concentration used in this experiment was 0·04 *M*; usually 0·025 *M* is used. This concentration does not however change the metabolism at 42° from its normal value.)

It will be seen that the chief rise in respiration occurs between 42 and 43°. Rabbits lose consciousness at about 43° (Marsh). Above this temperature little further rise in respiration occurs, though the aerobic glycolysis is markedly increased. This experiment suffers from the fact that a time effect may be in part responsible for the change of metabolism. We do know, however, that time of incubation has little effect on the metabolic quotients at 42°. At 45° however respiration does fall considerably with time. It would further appear that the main rise in glycolysis occurs when the respiration has reached its maximum. This could be well interpreted on the view that respiration and glycolysis have an initial common path. When the oxidative removal of some intermediary in the glycolytic chain has reached its maximum, then further increase in the rate of the initial reaction merely increases the rate of accumulation of lactic acid.

Further work is required on the effect of temperature on glycolysis and respiration in brain. It is clear, however, that a marked change in the type of metabolism occurs when we surpass the temperature limit which is compatible with the life of the whole animal.

SUMMARY.

1. The rates of respiration and of aerobic glycolysis in brain cortex at 42° are only slightly higher than these rates at 37°. At 45° however the respiration is increased by 100% above that at 37° and the aerobic glycolysis is increased many fold.

2. A similar abrupt rise in rate above 42° is also observed in the anaerobic glycolysis though this falls extremely rapidly at 45°.

3. The aerobic glycolysis at 45° is due to the disproportionately great increase in glycolysis in relation to the increase in respiration. There is no specific inhibition of the Pasteur effect, since the Meyerhof quotient is normal.

4. The results obtained show that the rates of carbohydrate catabolism in brain at various temperatures do not follow the Arrhenius law. The rate of catabolism rises very abruptly above 42°.

I wish to thank Sir Joseph Barcroft for his kind interest in this work.

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CCX. THE LACTIC DEHYDROGENASE OF ANIMAL TISSUES.

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MEYERHOF [1919] first observed the oxidation of lactic acid in presence of muscle and demonstrated the participation of a coenzyme in this oxidation. Szent-Györgyi and his co-workers [Szent-Györgyi, 1925, 1, 2; 1930; Banga *et al.* 1931; 1932; 1933] then studied in great detail the properties of the lactic coenzyme prepared from heart muscle and went a long way towards elucidating its chemical nature. Since the first description of the lactic dehydrogenase of animal tissues, some hundreds of papers have been written on the subject—most of which are discussed in Euler's monograph [1934]. But despite the vast accumulation of literature, definite information is lacking concerning the most fundamental characteristics of the lactic dehydrogenase, e.g. the nature of the oxidation product, the mechanism of the reaction with molecular oxygen, the quantitative distribution in various tissues, the role of the coenzyme etc. The difficulty which has impeded progress in the systematic investigation of this enzyme has been the inability of the various investigators to prepare an extract which could oxidize lactic acid aerobically with an appreciable velocity. In consequence, the study has been carried out mainly with minced tissue which allows of but a limited analysis; and in those cases where active extracts were prepared, the oxidation of lactic acid was measured only anaerobically in Thunberg tubes.

The purpose of the present investigation was to analyse the conditions necessary for the aerobic oxidation of lactic acid by the dehydrogenase system of animal tissues and to study in detail the properties and characteristics of the different components of the catalytic system. One of the significant conclusions which may be drawn from our study is that short-duration methylene blue experiments may present an unreliable account of the oxidation process, and that no study of dehydrogenases based entirely on simple methylene blue tube experiments can throw much light on the question of mechanism of reaction.

I. *Preparation of the components.*

Enzyme. The heart of the pig offers the most convenient source of the lactic dehydrogenase. The method of preparation however is generally applicable to the various tissues of any animal. Four hearts are divested of fat and connective tissue, passed through a Latapie mincer and washed exhaustively with tap water until the washings are haemoglobin-free. The washed mince is mixed with sand and 500 ml. *M*/50 phosphate buffer of *p*H 7 and ground to a fine paste in a mechanical mortar. The sand and insoluble debris are filtered off through muslin. The filtrate is centrifuged hard for 20 min. and the sediment resuspended in 200 ml. *M*/5 phosphate buffer of *p*H 7·2 (solution A) whilst the supernatant fluid is treated with 50 ml. *M*/2 acetate buffer of *p*H 4·6, and the flocculent precipitate centrifuged. The supernatant is discarded and the precipitate resuspended in 200 ml. *M*/5 phosphate buffer *p*H 7·2 (solution B). Both solutions contain an active lactic dehydrogenase. Usually the greater activity is found in solution B but the reverse has been observed in a few instances. Solutions A and B may be clarified extensively by prolonged centrifuging with little loss in activity. The enzyme is soluble but under certain conditions may be removed from solution by adsorption on impurities. The enzyme may be kept active for a long period in the form of a powder by drying *in vacuo* the centrifugates which are used to prepare solutions A and B respectively. In solution the enzyme retains the greater part of the original activity for 5–8 days when kept at 0°.

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[1] Beit Memorial Medical Research Fellow.

It is not necessary to use phosphate buffer exclusively. Bicarbonate, glycine and borate buffers are also satisfactory for most purposes.

Coenzyme. Baker's yeast is particularly rich in the coenzyme for the lactic dehydrogenase of animal tissues. The method of preparation follows closely that of Myrbäck [1933] for the preparation of cozymase. 9 kg. of yeast are crumbled into 6 litres of 2 % sulphuric acid maintained at 80°, and the mixture is vigorously stirred for 15 min. The yeast is filtered off on large Büchner funnels. 2 litres of saturated lead acetate (slight excess of reagent) are added to the filtrate and the precipitate filtered off and discarded. The filtrate is mixed with 700 ml. of saturated lead acetate, and 3·5 litres of hot saturated baryta are cautiously added to bring the *p*H to 9 (blue to thymolphthalein). The lead precipitate is filtered, washed thoroughly with water and decomposed in 3 litres of 0·6 *N* sulphuric acid. The lead sulphate is removed by filtration, and the filtrate is mixed with 300 ml. of a 25 % solution of phosphotungstic acid. Part of the precipitate is more or less colloidal and defies direct filtration. However, by impregnating the filter paper with a small amount of kieselguhr, all the precipitate is held back and the filtrate is perfectly clear. The filtration is extremely slow. The phosphotungstate precipitate is decomposed by suspending in 500 ml. of 10 % sulphuric acid and shaking the suspension vigorously with 2 volumes of a mixture of equal parts of amyl alcohol and ether. Usually an emulsion is formed which does not easily settle out. In that event, it must be centrifuged. The extraction is repeated with half the original volume of amyl alcohol and ether. The aqueous layer is filtered and the sulphuric acid removed with 1200 ml. of cold saturated baryta (final *p*H = 5). The deep yellow-brown colour observed in the previous stages is removed by the barium sulphate precipitate, and the filtrate now appears pale yellow. The volume is brought down by distillation *in vacuo* to 800 ml. and the slight precipitate filtered off. The *p*H is now adjusted to 7 with NaOH. This solution is stable for months when kept at 0°. It is advisable to use octyl alcohol as a preservative. 0·3 ml. or less of the coenzyme solution will activate 1 ml. of the lactic enzyme practically maximally.

The exact quantities used in different preparations are somewhat variable, and should be redetermined in each isolation experiment by small scale trials.

II. *The condition for linear oxidation.*

Suppose that given solutions of enzyme, coenzyme, lactate and methylene blue are mixed and the oxygen uptake measured. A rapid uptake is found to ensue for 5–10 min. and then the rate falls off sharply, so that at the end of 1 hour the final uptake is only slightly greater than at the end of the first few minutes. This effect can be explained in either of two ways: the enzyme is rapidly destroyed, or some product of the reaction inhibits the enzyme very strongly. It seemed more likely that the second alternative was the correct one and that the product of the oxidation of lactic acid, presumably pyruvic acid, was the agent responsible for the inhibition. Therefore if some means could be found of removing or fixing the pyruvic acid, the oxygen uptake should be linear for a considerable period. Cyanide is known to combine with ketones and Fig. 1 shows that in presence of this reagent, the rate of oxidation does not fall off appreciably before the first half hour. The rate of oxidation is increased as the strength of the cyanide is increased until a maximum concentration is reached—beyond which the enzyme is inhibited. Apparently the equilibrium point of the reaction

$$\text{Pyruvic acid} + \text{HCN} \rightleftharpoons \text{pyruvic cyanohydrin}$$

is not entirely to the right of the equation, and it is only with fairly high concentrations of cyanide that most of the pyruvic acid is fixed.

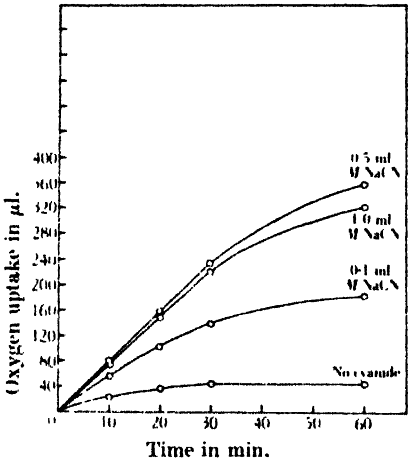

Fig. 1. Effect of cyanide on rate of oxidation of lactate. 1 ml. enzyme, 0·5 ml. coenzyme, 0·2 ml. 2 *M* lactate and 0·1 ml. 0·5 % methylene blue in each experimental manometer cup (water in the control cup). The lactate was placed in a dangling Keilin tube and was introduced after equilibration.

If the action of cyanide truly consists in combining with the pyruvic acid formed in the reaction, then the simultaneous addition of a large excess of pyruvate should abolish the cyanide effect whereas a small amount of added pyruvate should have little or no effect. Such is actually the case (Table I). It is interesting to note that 0·05 *M* pyruvate almost completely inhibits the oxidation of lactic acid in absence of fixing agent although this concentration of pyruvate in presence of cyanide is without effect.

Table I. *Effect of pyruvate in presence and absence of cyanide.*

Each manometer cup contained 1 ml. enzyme, 0·2 ml. 2 *M* lactate, 0·5 ml. coenzyme and 0·1 ml. 0·5 % methylene blue.

	Oxygen uptake in μl.	
	10 min.	20 min.
Enzyme system + 0·5 ml. *M* NaCN	79	149
Enzyme system + NaCN + 0·5 ml. 0·05 *M* pyruvate	74	157
Enzyme system + NaCN + 0·5 ml. 0·5 *M* pyruvate	19	39
Enzyme system + 0·5 ml. 0·05 *M* pyruvate	6	9

Hydroxylamine and hydrazine can also act in the capacity of pyruvic fixatives providing they are used in non-toxic concentrations (Table II). Dimedon, ammonia and semicarbazide have no action.

The experiments with cyanide have an interesting physiological significance. If the oxidation of lactate is to proceed linearly in the animal cell, there must be

Table II. *Hydroxylamine and hydrazine as pyruvic fixatives.*

Control contains 1·5 ml. enzyme, 0·3 ml. coenzyme, 0·2 ml. 2*M* lactate and 0·1 ml. 0·5 % methylene blue.

	Oxygen uptake in μl.	
	10 min.	30 min.
Control	15	24
+ 0·5 ml. *M*/10 hydroxylamine sulphate	50	116
+ 0·5 ml. *M* hydroxylamine sulphate	6	21
+ 1 ml. *M*/20 hydrazine sulphate	32	77
+ 1 ml. *M*/2 hydrazine sulphate	0	0

some mechanism for the rapid removal of pyruvic acid, otherwise the oxidation would be prevented. Peters & Thompson [1934] have shown that pyruvic acid accumulates in avitaminous pigeon brain and this pyruvic acid largely disappears upon the addition of vitamin B_1. Peters and his co-workers have in their past work also established that the rate of oxidation of lactate in avitaminous brain is subnormal. Through the courtesy of Prof. R. A. Peters we were able to test the action of pure vitamin B_1 on the isolated lactic system. The findings were negative as far as showing that the vitamin either prevents the accumulation of pyruvic acid or protects the lactic enzyme against the poisoning action of pyruvic acid.

III. *The product of oxidation.*

2:4-Dinitrophenylhydrazine does not precipitate pyruvic acid in presence of excess cyanide. However, the hydroxylamine compound of pyruvic acid is much more easily decomposed. The isolation experiment was therefore carried out with hydroxylamine as the fixing agent. 200 ml. of enzyme, 30 ml. of coenzyme, 20 ml. of 2*M* lactate, 30 ml. of *M* hydroxylamine and 5 ml. of 0·5 % methylene blue solution were mixed and aerated vigorously at 37° for 5 hours. After deproteinization with trichloroacetic acid and concentration *in vacuo* to 100 ml., 1 g. of 2:4-dinitrophenylhydrazine dissolved in 100 ml. of 2*N* HCl was added. The solution was allowed to stand at 0° for several hours before the crystalline precipitate was filtered off (yield 1·3 g.). After two recrystallizations from ethyl acetate the M.P. was found to be 215·5° which is identical with that of the hydrazone of pyruvic acid. The mixed M.P. was also 215·5°. The following are the analysis figures (Weiler). (C, 40·49; N, 21·07; H, 2·93 %. $C_9H_8O_6N_4$ requires C, 40·28; N, 20·90; H, 3·01 %.)

If pyruvate is the sole product of the oxidation of lactate, then for each mol. of lactate oxidized, 1/2 mol. of oxygen should be taken up. Fig. 2 shows the oxygen equivalence of lactate in presence of the enzyme system. Actually the limiting value of 1 atom of oxygen is not reached within 5 hours. The velocity of oxidation falls off very rapidly with decreasing concentration of substrate and in practice only as much as 70 % of the theoretical uptake is obtained before complete destruction of the enzyme sets in. Since racemic lactate was used, the theoretical oxygen requirement was calculated on the basis that only half the lactate is oxidized.

IV. p*H*.

The lactic enzyme is unusually sensitive to the constituents of buffer mixtures, and since a variety of buffers is necessary to cover the *p*H range from 4 to 13, the *p*H curve accordingly appears discontinuous (Fig. 3). It must be abundantly clear that deductions from the shapes of *p*H curves as to the dissociation of enzyme substrate complexes are not always reliable.

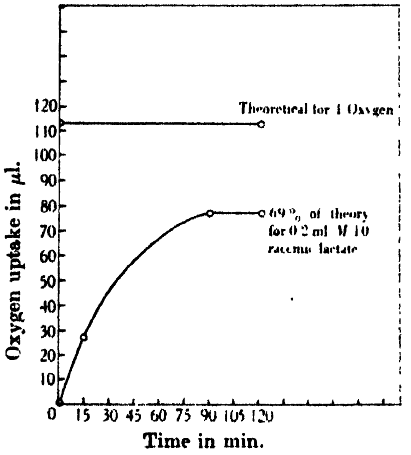

Fig. 2.

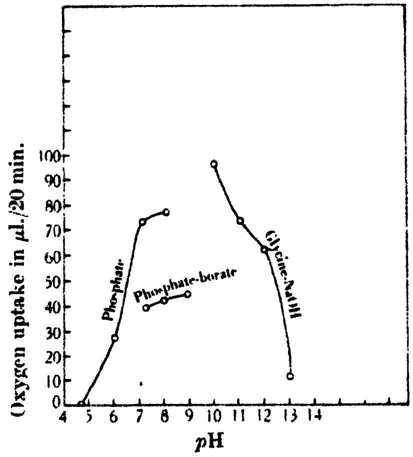

Fig. 3.

Fig. 2. The number of oxygen equivalents involved in the oxidation of lactate. The experimental manometer cup contained 1·5 ml. enzyme, 0·3 ml. coenzyme, 0·4 ml. *M* NaCN (neutral) and 0·1 ml. 1 % pyocyanine hydrochloride. The control without lactate was completely negative. The lactate solution was prepared from pure lactic acid syrup, the composition of which was checked by titration.

Fig. 3. The effect of *p*H. After precipitation with acetate buffer, the enzyme was suspended in water and divided into 12 aliquot portions each of which was brought to a given *p*H. The final volumes after the adjustment of *p*H were the same in all cases. The manometer cups contained 0·5 ml. neutralized enzyme, 2 ml. *M*/5 buffer, 0·3 ml. coenzyme, 0·2 ml. lactate (in Keilin cups) and 0·1 ml. 0·5 % methylene blue.

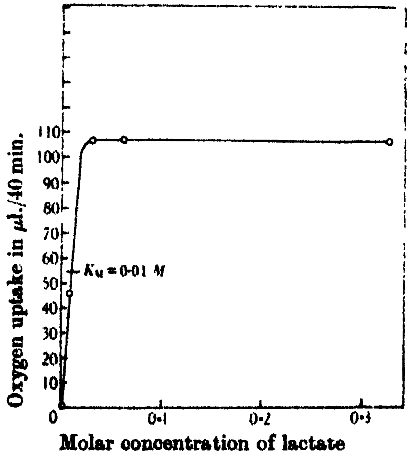

Fig. 4.

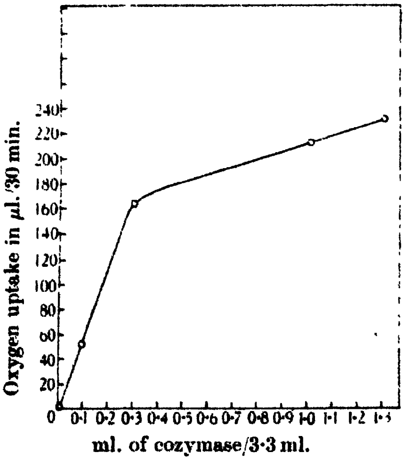

Fig. 5.

Fig. 4. Effect of the concentration of lactate on the rate of oxidation. 1·5 ml. enzyme, 0·3 ml. coenzyme, 0·1 ml. 0·5 % methylene blue and 0·4 ml. *M* NaCN were placed in all the experimental cups.

Fig. 5. Effect of the concentration of coenzyme on the rate of oxidation. 1·5 ml. enzyme, 0·1 ml. 0·5 % methylene blue, 0·4 ml. *M* NaCN and 0·2 ml. 2 *M* lactate were placed in all the experimental cups.

V. *Effect of concentration of lactate and coenzyme.*

Fig. 4 shows the dependence of the rate of oxidation on the concentration of lactate. Below $M/150$ the rate falls off rapidly, which means in effect that very small quantities of lactate are not oxidized with an appreciable velocity. The Michaelis constant is approximately $M/150$.

There is no critical concentration of coenzyme below which the rate of oxidation of lactate falls sharply (Fig. 5). The curve is linear at low concentrations of coenzyme and gradually approaches the value for the maximum rate of oxidation. It should be pointed out that an amount of coenzyme sufficient to saturate the enzyme in experiments with dilute methylene blue is insufficient when the methylene blue concentration is maximum for the activity of the enzyme.

VI. *Specificity of donator.*

The enzyme preparation in presence of the coenzyme oxidizes *l*(+)-lactate [180], β-hydroxypropionate [66], malate [200], fumarate [236], α- [24], β- [108] and γ- [6] hydroxybutyrate. The oxidations were tested for both anaerobically and aerobically with the same results. A comparison was made of the relative speeds of oxidation of these substrates in equimolar concentrations. The numbers in brackets refer to the oxygen uptake in μl./30 min. in presence of enzyme, coenzyme, methylene blue and cyanide. Fumarate, *l*(+)-lactate, malate and β-hydroxybutyrate are rapidly oxidized whereas α- and γ-hydroxybutyrates show only slight activity.

The following substances are not oxidized: *d*(−)-lactate, lactamide, malonate tartronate, glycerate, glycollate, mandellate, 3-phosphoglycerate, 2-phosphoglycerate, gluconate, α-glycerophosphate, acetate, alcohol, propionate, oxalate, acetaldehyde, pyruvate, citrate, serine, *iso*serine and glucose. It is interesting to note that lactamide ($CH_3.CH(OH).CONH_2$) and *iso*serine ($CH_2NH_2.CH(OH).COOH$) are not oxidized to the slightest extent although their chemical resemblance to lactic acid is very close.

The optical isomerides of lactic acid were prepared (*a*) by the morphine method and (*b*) by the resolution of the zinc ammonium salts. The *d*(−)-salt prepared by the two independent methods was inactive (Table III). We are grateful to the Distillers Co. Research Dept. for specimens of the pure *d*- and *l*-zinc ammonium salts.

Table III. *Oxidation of* d(−)- *and* l(+)-*lactate.*

Enzyme system: 1·5 ml. enzyme; 0·5 ml. coenzyme; 0·2 ml. 0·5% methylene blue and 0·4 ml. *M* NaCN (neutral).

	O_2 uptake in μl./30 min.	
	Morphine lactate	Zinc lactate
Enzyme system + 0·5 ml. *M*/5 *d*(−)-lactate	0	0
Enzyme system + 0·5 ml. *M*/5 *l*(+)-lactate	90	85

The lack of chemical similarity between fumarate and lactate made it improbable that the same enzyme system was responsible for the oxidation of both substrates. To test that point, the following experiment was performed. The times for the reduction of methylene blue in presence of the original enzyme system and the various substrates were measured (Table IV). The enzyme was then purified by treating thrice with kaolin, and the times of reduction again measured. If the same enzyme were involved in all these oxidations, the ratio of

Table IV. *Specificity of the enzyme.*

Each Thunberg tube contained 1 ml. enzyme, 0·2 ml. *M* substrate, 1 ml. water and 1 ml. 20 mg./100 ml. methylene blue (in side-bulb).

	Reduction time of methylene blue		
	Original preparation	Treated 3 × with kaolin	Treated 3 × with kieselguhr
Lactate	3 min. 15 sec.	6 min. 30 sec.	5 min.
Malate	2 „ 30 „	21 „	14 „
Fumarate	9 „	50 „	24 „
β-Hydroxypropionate	7 „ 30 „	120 „	∞
β-Hydroxybutyrate	4 „ 10 „	40 „	∞

the various rates should be the same regardless of the degree of purification of the enzyme preparation. The results accordingly indicate that the β-hydroxypropionate, fumarate, β-hydroxybutyrate and malate enzymes are not identical with the lactic enzyme. The same type of result is observed when the enzyme preparation is purified by treatment with kieselguhr. The extensive change after purification in the ratio of the rate of oxidation of lactate to the rate of oxidation of the other substrates is not a final proof of non-identity. The problem of the number of enzymes involved will be further considered in papers dealing with the fumarate and β-hydroxybutyrate enzymes.

A lactic enzyme can be easily prepared from an autolysed suspension of *Bact. coli* by the method of Stephenson [1928] and from baker's yeast by the method of Ogston & Green [1935]. A comparison was therefore made of the specificities of lactic enzyme preparations from different sources (Table V). The animal enzyme preparation is the only one to show complete optical specificity. *Bact. coli* enzyme preparation oxidizes $d(-)$-lactate more rapidly whereas the yeast preparation preferentially attacks $l(+)$-lactate. It is interesting to note that intact *Bact. coli* and yeast oxidize both isomerides at the same speed. That would suggest that there is an enzyme for each isomeride, and that in the method of preparation one is more easily destroyed than the other.

Table V. *Specificity of the lactic preparations from heart,* Bact. coli *and yeast.*

	Heart	*Bact. coli*	Yeast
$d(-)$-Hydroxypropionate	0	+ + + +	+
$l(+)$-Lactate	+ + + +	+ +	+ + + +
β-Hydroxypropionate	+ + +	0	+ +
Malate	+ + + +	0	+ + +
α-Hydroxybutyrate	+	+ + + +	+ + + +
β-Hydroxybutyrate	+ + + +	0	0
γ-Hydroxybutyrate	+	+	0

0 indicates no oxidation. + indicates oxidation.

The facts that the *Bact. coli* enzyme preparation does not oxidize β-hydroxypropionate or malate and that the yeast preparation does not oxidize β-hydroxybutyrate agree with the above conclusion that these three substrates are not oxidized by the particular enzyme of heart which catalyses the oxidation of lactate. The possibility however still remains that the specificities of lactic enzymes from various sources are not all the same and that the inability of the *Bact. coli* lactic preparation to oxidize malate may constitute no proof that the heart lactic enzyme similarly lacks this property.

VII. *Respiratory carriers and the reaction with molecular oxygen.*

A solution containing the enzyme, coenzyme and lactate does not react with molecular oxygen. The addition of a trace of methylene blue, pyocyanine or any other suitable oxidation-reduction indicator immediately catalyses the reaction with oxygen. The lactic dehydrogenase must therefore be considered as anaerobic in the sense that the reaction with oxygen is not direct but requires the intermediation of some respiratory carrier.

Table VI. *Carriers for the aerobic oxidation of lactate.*

Each manometer contained 1·2 ml. enzyme, 0·3 ml. coenzyme and 0·2 ml. 2 *M* lactate.

	µl. O_2/30 min.	Fixing agent
Control + NaCN	0	NaCN
+ 0·1 ml. 0·5 % methylene blue chloride	200	,,
+ 0·1 ml. 0·5 % pyocyanine hydrochloride	310	,,
+ 0·1 ml. 0·5 % flavin	152	,,
+ 1 ml. 10 % yellow pigment	20	,,
+ 0·1 ml. 0·5 % adrenaline	196	,,
Control + hydroxylamine	15	Hydroxylamine
+ 1 ml. 0·001 *M* cytochrome	15	,,
+ 0·5 ml. 0·5 % glutathione (S–S)	16	,,
+ 0·5 ml. 0·5 % ascorbic acid	25	,,
+ 0·5 ml. 0·5 % ascorbic acid (without lactate)	8	,,

Table VI contains a list of substances which were tested as respiratory carriers. With the exception of methylene blue and pyocyanine, these substances are normal constituents of animal cells. Flavin and adrenaline show high catalytic activity; yellow pigment is only slightly active, whereas cytochrome *c*, glutathione and ascorbic acid are inactive. Hydroxylamine must be used as the fixing agent when cytochrome, glutathione and ascorbic acid are studied since cyanide interferes with their catalytic activity (i.e. the activity in other systems in which they are known to function).

The variation of the rate of oxidation with change in the concentration of pyocyanine, methylene blue and flavin respectively is shown in Figs. 6, 7 and 8. Since the experiments with each carrier were done at different times and with enzyme preparations of different activities, the final limiting velocities are not identical in the three cases. It is interesting that in all cases at least half the maximum velocity is reached at a concentration of 0·25 mg. or less per 3·3 ml. Pyocyanine functions maximally in even lower concentration. With excess of pyocyanine inhibition of the enzyme sets in.

Wagner-Jauregg *et al.* [1934, 1, 2, 3; 1935] and Euler & Adler [1934; 1935] have proposed the theory that the yellow pigment (yellow enzyme) forms an essential component of many dehydrogenase systems. The experimental basis rests entirely on the following observations with methylene blue tubes. The time of decoloration of methylene blue by various enzyme systems is considerably shortened by the addition of yellow pigment. The same holds true for the reduction of flavin. These facts they interpreted to mean that the reduction of both methylene blue and flavin proceeds through the yellow pigment as an intermediary. Our experiments with the lactic system show that this conclusion of Wagner-Jauregg and of Euler and Adler is definitely incorrect. From Figs. 7 and 8 it is clear that, within limits, the higher the concentration of flavin or methylene blue, the more rapid is the rate of oxidation of lactate. When the concentrations become very small—of the order of magnitude of the concentrations used in methylene blue experiments ($< 50\gamma$)—then the catalytic activities of both flavin and methylene blue become enormously reduced and approach zero. In effect that means that very small quantities of both methylene blue and flavin are reduced extremely slowly by the lactic dehydrogenase system. Suppose that a substance could be found with a very low Michaelis constant (i.e. it was able to function maximally at high dilutions) which in the reduced form could reduce methylene blue or flavin. Then the addition of such a substance to the lactic dehydrogenase system or any of the other coenzyme systems would result in a more rapid reduction of flavin or methylene blue when they were present in small concentration (< 50γ) but not when they were in a sufficiently

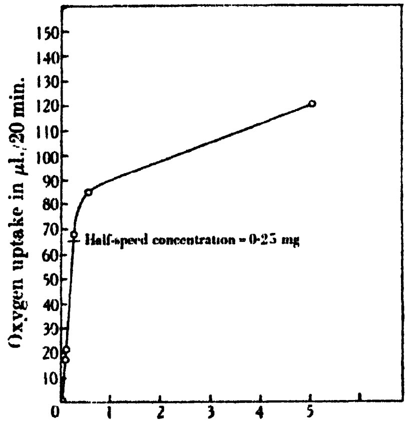

Fig. 6.

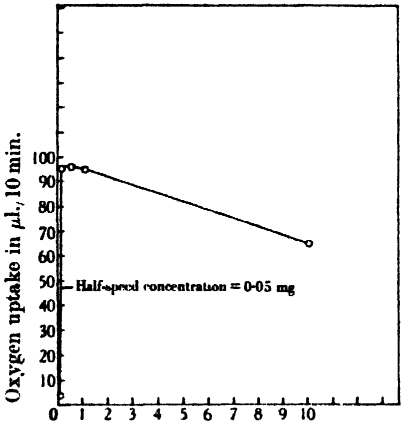

Fig. 7.

Fig. 6. Effect of concentration of methylene blue chloride on the rate of oxidation. 1·4 ml. enzyme, 0·3 ml. coenzyme, 0·4 ml. *M* NaCN and 0·2 ml. 2*M* lactate in all the experimental cups.

Fig. 7. Effect of concentration of pyocyanine hydrochloride on the rate of oxidation. 1·4 ml. enzyme, 0·3 ml. coenzyme, 0·2 ml. 2*M* lactate and 0·4 ml. *M* NaCN in all the experimental cups.

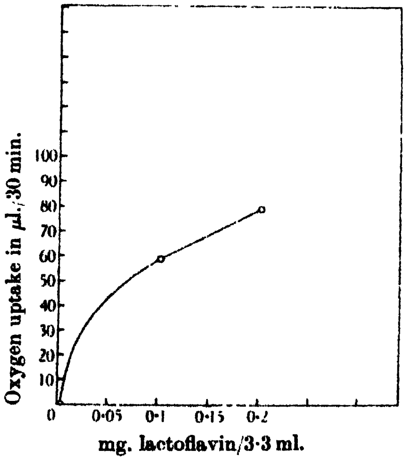

Fig. 8. Effect of concentration of lactoflavin on rate of oxidation. 1·5 ml. enzyme, 0·3 ml. coenzyme, 0·4 ml. *M* NaCN and 0·2 ml. 2*M* lactate in all the experimental cups.

high concentration to allow the enzyme to work at maximum efficiency. Yellow pigment has the properties of this hypothetical substance. It can be reduced by the dehydrogenase system and in the reduced form can reduce in turn either flavin or methylene blue. Furthermore, the Michaelis constant is very low indeed, which means that the rate of reduction of yellow pigment is constant down to high dilution. The yellow pigment effect must therefore be interpreted in terms of affinity constants and offers no support of the theory that methylene blue or flavin is not reduced directly by coenzyme systems but only through the intermediation of the yellow pigment.

The curious fact is that even a large excess of yellow pigment shows a slight catalytic activity (Table VII) hardly comparable with the activity in presence of excess of either flavin or methylene blue. Furthermore, in aerobic experiments, the addition of yellow pigment does not increase the flavin effect over a wide range of flavin concentrations. This comparative inactivity of yellow pigment with the lactic system is rather unexpected and requires further study. The pigment was prepared from bottom yeast by the method of Warburg & Christian [1933]. We are indebted to Prof. O. Warburg for his generosity in placing at our disposal a sample of a relatively pure preparation. Both our preparation and the authentic gave the same results.

Table VII. *Effect of yellow pigment on enzyme purified with kaolin.*

1·5 ml. enzyme, 0·3 ml. coenzyme, 0·3 ml. M NaCN (neutral) and 0·2 ml. $2M$ lactate in each experiment.

	μl. O_2/30 min.
Control	0
+ 0·1 ml. 0·5% methylene blue	63
+ 0·5 ml. 5% yellow pigment	19
+ yellow pigment + methylene blue	67
+ 0·5 ml. 0·5% lactoflavin	50
+ lactoflavin + yellow pigment	48

The analysis of the adrenaline effect will be dealt with in a separate communication by Green and Brosteaux. The essential facts may be summarized as follows. The lactic system reacts vigorously with oxygen in presence of adrenaline. If any of the components of the lactic system are omitted, e.g. the coenzyme or lactate, the adrenaline effect disappears. The results of many control experiments show conclusively that adrenaline is acting as a respiratory catalyst in the lactic system. The mechanism of the catalysis is very complex. Adrenaline is the precursor of the actual catalytic system. Upon addition of adrenaline to the complete lactic system, a red colour rapidly develops and persists throughout the reaction. This red substance can be reduced and oxidized reversibly.

There is an additional factor concerned in the adrenaline effect with the lactic system. Its function is to catalyse the aerobic oxidation of adrenaline to the red substance. This oxidation depends upon the simultaneous oxidation of lactate to pyruvate. In absence of the specific factor, adrenaline has no action.

Any of the dehydrogenase systems which require the lactic coenzyme can utilize adrenaline as a respiration carrier. The adrenaline effect seems to be a general property of lactic coenzyme systems.

It is impossible from experiments *in vitro* to decide which of the natural carriers of animal source are involved in the oxidation of lactate within the living cell. There is presumably sufficient of flavin, adrenaline and yellow pigment in most cells to account for the observed rate of oxidation of lactate. But in the absence of experiments carried out on intact cells, no definite conclusions can be drawn. The cyanide-insensitivity of the isolated lactate system in contrast to the cyanide-sensitivity of the lactate oxidation in tissue slices (Table VIII) may indicate that some carrier other than flavin, yellow pigment and adrenaline is involved physiologically. Another possibility remains that it is not the oxidation of lactate but that of pyruvate which is poisoned by dilute cyanide. The accumulation of pyruvate in presence of the reagent will thereby prevent the oxidation of lactate.

Table VIII. *Cyanide inhibition of lactate oxidation in tissue slices.**

Pigeon kidney	Q_{O_2}	Rabbit brain	Q_{O_2}
Control	- 10·5	Control	- 5·9
+ $M/500$ cyanide	- 2·7	+ $M/500$ cyanide	- 1·3
+ $M/125$ lactate	- 18·4	+ $M/125$ lactate	- 10·9
+ lactate + cyanide	- 2·9	+ lactate + cyanide	- 1·7
		+ lactate + 0·5 mg. lactoflavin	- 10·5
		+ lactate + lactoflavin + cyanide	- 1·0

* These experiments were kindly carried out for us by Dr L. F. Leloir.

A comparison of the lactic dehydrogenase of animal cells with that of yeast reveals some very instructive differences. The former requires a coenzyme and reacts with flavin but not with cytochrome. The latter requires no coenzyme, and reacts with cytochrome but not with flavin [Ogston & Green, 1935]. It may be taken as a general rule that cytochrome is inactive in systems requiring a coenzyme. Conversely flavin is inactive in systems which do not require a coenzyme. There appears to be a fundamental distinction between the coenzyme-flavin systems and the cytochrome systems. Adrenaline shows the same behaviour as flavin in respect of its activity with the coenzyme and non-coenzyme systems. The three dehydrogenase systems which are known to react with molecular oxygen *via* cytochrome (yeast lactate, succinate and α-glycerophosphate of animal source) do not require a coenzyme. Conversely the dehydrogenase systems that react with flavin or yellow pigment (hexosemonophosphate, lactate, malate, glucose and alcohol) are incapable of reducing cytochrome with any appreciable velocity. The explanation of these curious facts will be dealt with in later sections.

VIII. *Reduced coenzyme and the mechanism of coenzyme action.*

The mechanism of the action of coenzymes in enzymic dehydrogenations—a long-standing biochemical puzzle—has become remarkably clear as the result of the recent brilliant study by Warburg *et al.* [1935]. Very convincing evidence has been adduced to show that the following series of events takes place in the reaction between hexosemonophosphate and oxygen:

(1) hexosemonophosphate + coenzyme $\xrightarrow{\text{enzyme}}$ phosphohexonate + reduced coenzyme,
(2) reduced coenzyme + yellow pigment $\longrightarrow$ coenzyme + leuco-yellow pigment,
(3) leuco-yellow pigment + $\frac{1}{2}O_2$ $\longrightarrow$ yellow pigment.

The coenzyme is therefore to be considered as a highly specific carrier linking the dehydrogenase with the yellow pigment. The reversible reduction of the coenzyme can be effected not only enzymically but also with hydrosulphite. Reduced coenzyme is not autoxidizable. In the reconstructed system, the oxidation is brought about by yellow pigment.

The researches of Warburg *et al.* were confined to the coenzyme of red blood cells which is not identical with the coenzyme of lactic dehydrogenase (cf.

section X). It was therefore of interest to inquire how general is the behaviour of the Warburg coenzyme and to what extent the above scheme is applicable to the lactic enzyme. The results of the inquiry indicate that the mechanism of the action of the lactic enzyme closely parallels that of the Warburg coenzyme.

The lactic coenzyme prepared as in section I does not reduce methylene blue or flavin. However, by adding hydrosulphite to a neutral buffered solution of the coenzyme and by then oxidizing excess hydrosulphite in a stream of air, a solution of coenzyme is obtained which reduces methylene blue and flavin; e.g. 1 ml. reduced coenzyme reduced 0·2 ml. 0·02 % methylene blue in 10 min. and 0·1 ml. 0·05 % lactoflavin in 80 min. A trace of yellow pigment decreased the times of reduction of methylene blue and flavin to 1·5 and 10 min. respectively. Controls of buffer and of alkali-inactivated coenzyme treated in exactly the same way failed to show this reducing power. The presumption is therefore reasonable that hydrosulphite reduces the coenzyme and that reduced coenzyme is the agent responsible for the reduction of methylene blue. The catalytic effect of yellow pigment offers additional confirmation of this view.

The lactic coenzyme can also be reduced by the complete dehydrogenase system. If the coenzyme is incubated with a mixture of enzyme, lactate and cyanide for several minutes, and the mixture is then boiled and filtered through kieselguhr, a solution of reduced coenzyme is obtained which shows the same properties as that of reduced coenzyme prepared by the hydrosulphite method.

Assuming that Warburg's theory applies to the lactic coenzyme, it follows that any carrier which is active with the whole dehydrogenase system should be reduced by reduced coenzyme and conversely carriers which have no activity should not be reduced with any appreciable velocity. This prediction is in fact fulfilled. Reduced coenzyme can reduce methylene blye, pyocyanine, flavin, the red oxidation product of adrenaline and yellow pigment but has no action on cytochrome *c*. The evidence is therefore suggestive that in the reaction between lactate and oxygen, the coenzyme is reduced, and in turn reduces the carrier which finally reacts with molecular oxygen. The problem—which carriers of animal tissues are active with coenzyme dehydrogenase systems—simplifies to the problem—which carriers are reduced by the reduced coenzyme. It is predicable that all systems which require the lactic coenzyme will react with flavin, yellow pigment or adrenaline but not with cytochrome. The possibility of interaction is determined not by the enzyme but by the coenzyme which is the factor in common.

The suggestion has been made that the lactic dehydrogenase of yeast although independent of a soluble coenzyme is rigidly bound to its coenzyme and hence cannot be resolved into the two components by the ordinary methods. The suggestion is not tenable in view of the fact that the reduced coenzyme does not reduce cytochrome whereas the yeast enzyme reacts vigorously with molecular oxygen in presence of cytochrome. Similarly the reduced lactic coenzyme reduces flavin and yellow pigment whereas these two substances have no catalytic action on the yeast lactic system.

Several properties of reduced coenzyme are worth mentioning. Acid solutions rapidly destroy reduced coenzyme, although strong alkali at 100° has no effect. The reverse is true for the oxidized coenzyme. The reduced coenzyme shows a broad adsorption band at 330–340 mμ in the ultraviolet whereas the oxidized coenzyme shows only small absorption in this region. Dr E. van Heyningen kindly carried out the ultraviolet measurements. Lastly, reduced coenzyme is as active as oxidized coenzyme with the dehydrogenase system. This fact is consistent with the theory of the mechanism of coenzyme action.

Given an excess of enzyme, the limiting factor in the oxidation of lactate should be the rate of reaction between reduced coenzyme and the carrier. Since the enzyme is not involved in this process, it follows that reduced coenzyme should react with flavin or methylene blue at the same speed as in presence of the enzyme. Actually such is not the case. The rate of reduction of methylene blue by reduced coenzyme is much slower in absence than in presence of the enzyme. The ratio of activities is 1 : 10 in presence of yellow pigment and 1 : 90 in absence of yellow pigment. Furthermore, yellow pigment enormously accelerates the rate of reaction between reduced coenzyme and methylene blue or flavin but not so in the presence of the enzyme. These two discrepancies may mean that the carrier theory of coenzyme action requires modification in part.

IX. *Reversibility.*

The enzymic oxidation of hypoxanthine to uric acid, of hydrogen gas to hydrogen ion or of succinate to fumarate is reversible under suitable conditions [Green, 1934; Green & Stickland, 1934; Filitti, 1934; Quastel & Whetham, 1924; Borsook & Schott, 1931]. The equilibrium potential of the system hypoxanthine-xanthine oxidase-uric acid (to take one example) can be measured by determining to what extent this system reacts with benzylviologen. The degree of reduction of the indicator is a direct measure of the potential corresponding to the equilibrium point.

The question of the reversibility of a dehydrogenase system involving a coenzyme raises certain theoretical considerations. In the case of the xanthine oxidase, there is a direct interaction between hypoxanthine and benzylviologen, and between reduced benzylviologen and uric acid. This simple condition does not apply to the lactic dehydrogenase system of animal tissues. The reaction between lactate and the indicator is not direct but proceeds through the intermediation of the coenzyme. For a reversible state to be established, the following steps must be reversible:

(1) lactate + coenzyme $\rightleftharpoons$ pyruvate + reduced coenzyme,
(2) reduced coenzyme + indicator $\rightleftharpoons$ coenzyme + reduced indicator.

Clearly the reversibility of the oxidation of lactate to pyruvate will depend on whether the coenzyme system can come into equilibrium (*a*) with the enzyme system and (*b*) with the indicator system.

The potential of a mixture of lactate, pyruvate, coenzyme and indicator will be determined by the system in highest concentration. In practice the concentrations of lactate and pyruvate are arranged to be in great excess over those of coenzyme or indicator. Hence if an equilibrium potential can be measured, it will refer to the potential of the system lactate-pyruvate and will not be governed by either the coenzyme or indicator system.

Banga *et al.* [1933] claimed to have demonstrated the thermodynamic reversibility of the lactate system of pig's heart muscle. Using Janus green as the indicator of the equilibrium, they found E_0' at pH 7·0 to be −0·181 v. We have attempted to reoxidize leuco-Janus green with pyruvate in presence of the enzyme system but the results thus far have been negative. Janus green is reduced with extreme slowness, and is therefore of questionable value for demonstrating reversibility. Lactoflavin however is reduced quite rapidly and appears to form an equilibrium with the lactate system. In presence of excess lactate, lactoflavin is reduced practically to completion whilst the leuco-form can be reoxidized by excess pyruvate.

Table IX. *Reduction of oxidation-reduction indicators by lactic system and reduced coenzyme at* p*H 7·0.*

Experiments were carried out anaerobically in Thunberg tubes.

	E_0' at *p*H 7·0 in v.	Lactic system	Reduced coenzyme
Methylene blue	+0·011	Reduced completely	Reduced completely
Ethyl capri blue	-0·060	,,	,,
Nile blue	-0·142	,,	,,
Lactoflavin	-0·210	,,	,,
Yellow pigment	?	,,	,,
Janus green (red to colourless)	-0·258	,,	,,
Benzylviologen	-0·359	No reduction	No reduction

Table IX compares the indicators reduced by the enzyme system plus coenzyme and by the reduced coenzyme alone. The parallelism is very striking. The possibility presents itself that the equilibrium observed between the enzyme system and lactoflavin may concern the coenzyme system primarily and may not be a measure of the potential of the lactate-pyruvate system.

The usual method of fixing the ratio of lactate to pyruvate and then determining the potential either colorimetrically or electrometrically is not applicable to the lactate system of heart. Since traces of pyruvate inhibit almost completely oxidation of lactate, the measurements with mixtures are concerned with kinetics and not with thermodynamic equilibria. The question of the reversibility of the lactate system of heart must be reconsidered in this new light. The testing of the reversibility of the lactic systems of bacteria apparently does not involve these difficulties [cf. Barron & Hastings, 1934; Wurmser & Mayer-Reich, 1933].

Reduced coenzyme can reduce flavin to completion. The addition of oxidized coenzyme does not result in reoxidation of leuco-flavin. If the lactate-pyruvate system were reversible, we should expect that the ratio of reduced coenzyme to oxidized coenzyme would determine the ratio of reduced to oxidized flavin.

X. *Specificity of coenzyme.*

Identity of heart and yeast lactic coenzymes. A lactic coenzyme can be prepared from heart muscle by the method of Banga *et al.* [1933]. The fact that the coenzymes from heart and yeast can replace one another in the lactic system of animal tissues constitutes little or no proof of their possible identity. The sole alternative to isolating the respective coenzymes and establishing their identity by direct chemical means is an extensive comparison of the chemical and physical properties of the two coenzymes.

Given the respective coenzyme solutions in known catalytic strengths, the original ratio of activities should persist through a variety of parallel treatments if the coenzymes are identical. The constancy of the ratio is the measure of identity. Table X contains a summary of the comparison. The times in minutes refer to the reduction of methylene blue unless otherwise stated. The original ratio of 0·8 varies between the limits of 0·5 and 1·0. Bearing in mind that the impurities in the two preparations are different and that they may influence to some extent the behaviour of the coenzyme, the small variations in the ratio cannot be considered significant. There is little doubt that the chemical and physical properties of the two coenzymes are very similar, and we may reasonably assume that they are identical.

Table X. *Comparison of heart and yeast lactic coenzyme.*

The quantity of 0·02% methylene blue used with the enzyme system was 1 ml. and with the reduced coenzyme 0·2 ml.

	Heart min.	Yeast min.	Amount of coenzyme in ml.	Ratio
(1) With enzyme system.				
Original coenzyme solutions	4	5	0·2	0·8
Treated in cold with alkali	5·5	5·5	0·5	1·0
Treated with boiling alkali	35	60	0·5	0·6
(2) Without enzyme system.				
Original reduced coenzyme	18	37	0·8	0·5
+ yellow pigment	2	4·5	0·8	0·6
Treated with acid in cold (tested with yellow pigment)	50	100	2·0	0·5
Reduction of pyocyanine by original reduced coenzyme	6	10	0·8	0·6

Identity with the hexosemonophosphate coenzyme. Through the courtesy of Prof. Keilin and Prof. Warburg we were able to test the activity of a highly purified preparation of the hexosemonophosphate coenzyme in the lactic dehydrogenase system, cf. Table XI. The result was completely negative. It is interesting that the malate and β-hydroxybutyrate systems are also not activated by the hexosemonophosphate coenzyme (Table XII). Considering the similarity in the chemical constitution and in the mode of action of the lactic and hexosemonophosphate coenzymes, the observed specificity is most extraordinary.

Table XI. *The effect of Warburg coenzyme.*

Control lactic system: 1·5 ml. enzyme, 0·2 ml. 0·5% methylene blue, 0·4 ml. M NaCN and 0·2 ml. $2M$ lactate. Control hexosemonophosphate system; 1 ml. dialysed yeast juice, 0·2 ml. 0·5% methylene blue, 0·2 ml. $0{\cdot}33M$ hexosemonophosphate.

	μl. O_2/30 min.
Control lactic system without coenzyme	10
+ 0·5 ml. lactic coenzyme	220
+ 0·5 ml. Warburg coenzyme	10
Control hexosemonophosphate system without coenzyme	0
+ 0·5 ml. lactic coenzyme	0
+ 0·5 ml. Warburg coenzyme	171

Table XII. *Effect of Warburg coenzyme on oxidation of malate and β-hydroxybutyrate.*

1 ml. 0·02% methylene blue, 0·2 ml. $2M$ substrate, 1 ml. lactic enzyme preparation.

	Reduction time of methylene blue min.
Enzyme + malate	∞
Enzyme + malate + 0·5 ml. lactic coenzyme	2·5
Enzyme + malate + 0·5 ml. Warburg coenzyme	∞
Enzyme + malate + β-hydroxybutyrate	∞
Enzyme + malate + 0·5 ml. lactic coenzyme	2·7
Enzyme + malate + 0·5 ml. Warburg coenzyme	∞

Identity with cozymase. Needham & Green (unpublished experiments) have made a systematic comparison of the activities of various coenzyme preparations from yeast and heart with regard to (1) alcoholic fermentation and (2) the oxidation of lactate by the animal dehydrogenase system. In general the ratio of the activities in the two processes was constant for preparations derived from various sources and subjected to various chemical and physical treatments. There was however one notable exception, viz. a coenzyme solution which was very active in catalysing fermentation but which apparently had little effect on the oxidation of lactate.

Both cozymase and the coenzyme of lactic oxidation are rapidly destroyed by alkali. On reduction with hydrosulphite, the lactic coenzyme becomes insensitive to alkaline treatment. Needham & Green tested reduced coenzyme boiled with alkali as the coenzyme of fermentation and found the activity unimpaired. This striking similarity in the chemical properties of lactic coenzyme and cozymase suggests the identity of the two coenzymes.

Needham & Green considered the relative magnitudes of the cozymase and lactic coenzyme catalyses. They found that with excess of enzyme in each case (and with the same amount of coenzyme) about 20 mols. of CO_2 are liberated in fermentation for 1 mol. of oxygen absorbed in lactate oxidation during the same period. This disparity in rates contrasts with the similarity in rates observed when a limited amount of coenzyme is used with excess of the various dehydrogenase systems which require in common the lactic coenzyme. The suggestion is therefore obvious that although the lactic coenzyme and cozymase are almost invariably concomitant and may be associated in the same molecule, yet the mechanisms of their respective actions are different. Possibly the one case where the activity of cozymase was not associated with significant lactic coenzyme activity may be explained on the basis that the original coenzyme molecule underwent some change whereby one function was impaired and not the other.

For the present in view of these results of Needham & Green, it is preferable not to designate the lactic coenzyme as cozymase. The final decision of nomenclature must be deferred until the question of identity is settled conclusively.

Synthetic coenzymes. Warburg *et al.* [1935] demonstrated that the hexosemonophosphate coenzyme contains 2 mols. of ribose, 3 of phosphoric acid and 1 of adenine and nicotinamide. In the reversible oxidation and reduction of the coenzyme, it is the nicotinamide moiety which is directly involved. Warburg *et al.* showed that trigonelline (methylbetaine of nicotinic acid) behaves like the coenzyme in many respects, e.g. it can be reversibly reduced with hydrosulphite and in the reduced form is not autoxidizable. Furthermore, yellow pigment can oxidize the reduced form. More recently, Karrer & Warburg [1936] have prepared several nicotinic derivatives and have tested their ability to be reduced by hydrosulphite and to be oxidized by yellow pigment.

The question arose whether any simple nicotinic acid derivative could replace the coenzyme in the complete dehydrogenase system. The following substances were tested: nicotinic acid, nicotinamide, nicotine, diethyl ester of nicotinamide (coramine), trigonelline and the methiodides of the first four mentioned. There was no indication of any activity except for a very slight acceleration by trigonelline of the reduction of methylene blue by the lactic enzyme system. The effect however was far too small to merit serious consideration. It appears therefore that the lactic coenzyme is extremely specific and is not replaceable by substances of similar chemical behaviour and constitution.

XI. *Purification.*

Solutions A and B of lactic enzyme prepared according to section I may be extensively purified by the following treatments with either kaolin or kieselguhr. 20 ml. of enzyme solution are shaken vigorously with 1 g. of the adsorbent and then centrifuged. After two or three such treatments, the enzyme solution

Table XIII. *Purification of lactic enzyme.*

Tests were made with 1 ml. enzyme, 0·5 ml. coenzyme, 0·4 ml. *M* NaCN (neutral), 0·2 ml. 0·5 % methylene blue and 0·2 ml. 2 *M* lactate.

The preparations of solutions A and B are described in section I.

Solution A.	Q_{O_2}	Solution B.	Q_{O_2}
Original after centrifuging	167	Original after centrifuging	10
Treated twice with kieselguhr	457	Treated thrice with kaolin	18
		Treated thrice with kieselguhr	34

becomes almost water clear. Table XIII shows the extent of the purification as measured by the Q_{O_2}. The absolute value of the Q_{O_2} depends upon how fresh are the hearts used in the preparation of the enzyme and how quickly the experiment is carried out after the preparation is completed. The greatest fall in activity is in the first 24 hours. After that period the loss in activity is much more gradual. Often solution A is less active than B. The ratio of activities is variable and depends upon the particular heart.

The purified enzyme is no longer precipitated completely by acetic acid and furthermore undergoes rapid destruction in presence of acetic acid. No way has yet been found of obtaining the purified enzyme in the dry form.

When the Q_{O_2} determinations are made, the use of phosphate buffers must be avoided. The precipitates for solutions A and B respectively are suspended in water and the *p*H adjusted to 7·0 with dilute NaOH. This procedure eliminates the use of phosphate which introduces a large error in determining the dry weight of the enzyme preparation.

Table XIV. *Inhibitors of lactic enzyme.*

All the experiments were carried out with cyanide as the fixative except in the case of pyruvate.

	Final concentration *M*	% inhibition
Octyl alcohol	Saturated aqueous solution	0
Ethylurethane	0·16	33
Pyrophosphate	0·08	0
Cyanide	0·33	0
Arsenious acid	0·33	0
Iodoacetate	0·02	0
Pyruvate	0·04	100
Tartronate	0·03	60
d(–)-Lactate	0·03	0

XII. *Inhibitors.*

None of the usual respiration inhibitors affects the lactic enzyme appreciably (Table XIV). Urethane in high concentration produces only 30 % inhibition. Pyruvate and tartronate inhibit by virtue of their ability to be adsorbed on the active enzyme surface, thus preferentially displacing lactate. It is interesting that *d*(–)-lactate does not show this phenomenon of competitive inhibition. That rather indicates that even the purely physical process of the adsorption of a substrate on the enzyme molecule may involve stereochemical specificity.

XIII. *Distribution of the enzyme.*

The quantitative estimation of the amount of any particular enzyme present in a tissue involves difficulties which at the moment seem insuperable. Using the tissue slice technique, the difference between the Q_{O_2} in presence and in absence of lactate provides the measure of the amount of enzyme present. The results however are very unsatisfactory for the following reasons: (1) the tissue may be saturated with lactate and hence the addition of more lactate produces no increase; (2) the missing component of the lactic system may not be the enzyme but the coenzyme. Hence with slices like those of muscle which allow the coenzyme to leak out into the surrounding medium, the fact that addition of lactate does not increase the Q_{O_2} has no bearing on the question whether there is any lactic enzyme present.

We have adopted the following method for estimating the amount of lactic enzyme present in various tissues. The organs of freshly killed animals only are used. After coarse mincing with scissors, the tissue is washed exhaustively with tap water to remove soluble components and then thoroughly ground with sand and a known volume of $M/100$ phosphate buffer pH 7. The success of the method depends entirely on the completeness of the grinding process. The sand and insoluble debris are filtered off through muslin; the filtrate is centrifuged and tested directly. The entire procedure is a matter of 10–15 min. The filtrate contains only the enzyme and must be supplemented with coenzyme, lactate, methylene blue and cyanide in order to reconstruct the whole system. A control with all the components except lactate must be carried out to correct for the blank of the enzyme.

The Q_{O_2} refers to the oxygen uptake in μl./hour of 1 mg. dry weight of the enzyme preparation. The phosphate of the buffer must be allowed for in determining the dry weight. The question arises whether the Q_{O_2} should be calculated on the basis of the original dry weight of tissue which corresponds to 1 mg. dry weight of enzyme. The Q_{O_2} values for tissues calculated on the basis of the original tissue dry weight are of course numerically different from the standard Q_{O_2} values. However, the relative activities of the enzyme in the various tissues are approximately the same regardless of the method of calculation, cf. Table XV.

Table XV.

	O_2 uptake in μl./hour/mg. dry wt. of tissue	O_2 uptake in μl./hour/mg. dry wt. of extract	Ratio
Muscle	1·1	6·7	6
Heart	1·3	7·4	6
Brain	2·0	12·2	6
Liver	0·2	1·0	5

Table XVI. *The distribution of the lactic enzyme in rat, rabbit and pigeon.*

$Q_{O_2\ \mathrm{lactate}}$ = oxygen uptake in μl./hour/mg. dry weight of enzyme extract.

Enzyme extract tested with 0·5 ml. coenzyme, 0·3 ml. M NaCN (neutral), 0·2 ml. 0·5% methylene blue and 0·2 ml. $2M$ lactate.

These values of the $Q_{O_2\ \mathrm{lactate}}$ are the maxima found in repeat experiments.

	Rat	Rabbit	Pigeon
Kidney	79	9	3·1
Heart	111	15	4·5
Brain	73	32	0·4
Muscle	28	30	7·3
Lung	—	3·4	0·9
Liver	—	1·2	1·8

The distribution of the enzyme in various animals is shown in Table XVI. There can be little doubt that the lactic enzyme is an essential part of the enzymic equipment of all animal cells. It is interesting that the highest concentrations of the enzyme were found in the tissues of the rat compared with those of rabbit and pigeon. This is to be expected on the basis of the Q_{O_2}/body weight relation.

The tissues of the rabbit fail to yield active extracts of the lactic enzyme when ground with sand and distilled water. Activity is only obtained when salts, preferably phosphates, are present in the surrounding fluid. This effect of phosphate is confined to the tissues of the rabbit. In the absence of phosphate no activity can be found either in the residue or in the extract. It appears that enzyme destruction occurs under these conditions.

XIV. *The classification of dehydrogenases.*

The experimental investigations of Warburg & Christian [1931: 1933], Keilin & Hartree [1936], Euler & Adler [1934: 1935], Wagner-Jauregg *et al.* [1934; 1935], Ogston & Green [1935] and Green [1936] have provided the groundwork for a systematic classification of dehydrogenase systems.

I. *Aerobic oxidases:*

Characteristics: react directly with molecular oxygen: produce H_2O_2; do not require coenzyme.

Examples: uricase, amino-acid oxidase, xanthine oxidase.

II. *Cytochrome dehydrogenase systems:*

Characteristics: do not react directly with molecular oxygen; can react through cytochrome but not flavin or yellow pigment; do not require coenzyme.

Examples: α-glycerophosphate, succinate, lactate (yeast).

III. *Coenzyme dehydrogenase systems:*

Characteristics: do not react directly with molecular oxygen; react with flavin or yellow pigment but not cytochrome: require a coenzyme.

A. Hexosemonophosphate coenzyme systems:

Examples: glucose (liver) and hexosemonophosphate of yeast or red blood cells.

B. Lactic coenzyme systems:

Examples: lactate (animal tissues), malate, fumarate, β-hydroxybutyrate, glucose (liver).

The glucose system of liver is the only one known to work with either of two coenzymes [Euler & Adler, 1935].

SUMMARY.

The lactic dehydrogenase system of animal tissues comprises the following components: enzyme, coenzyme, lactate and carrier.

The product of oxidation—pyruvic acid—inhibits almost completely the oxidation of lactic acid. Ketone-fixing agents, such as cyanide and hydroxylamine, combine with pyruvic acid and allow the oxidation of lactic acid to proceed linearly for a considerable period.

The effects of *p*H and of concentration of coenzyme and substrate upon the rate of oxidation have been studied.

The lactic enzyme is found associated with the enzymes for the oxidation of malate, fumarate, β-hydroxypropionate and β-hydroxybutyrate. Methods of separating partially the lactic enzyme from the others are described.

The lactic enzyme catalyses the oxidation of *l*(+)-lactate to pyruvic acid which can be isolated as the 2:4-dinitrophenylhydrazine derivative. *d*(−)-Lactate, lactamide and *iso*serine are not oxidized.

Among the natural carriers, flavin, adrenaline and to a slight extent yellow pigment (flavoprotein or yellow enzyme) can function as carriers for the reaction with oxygen. Cytochrome, ascorbic acid and glutathione are inactive.

The lactic coenzyme can be reduced with hydrosulphite or by the enzyme system. Reduced coenzyme can reduce flavin, methylene blue, yellow pigment and the red substance formed from adrenaline. It does not however reduce cytochrome. Reduced coenzyme is unstable in acid solution but not in alkaline.

The Warburg scheme of coenzyme action is found to apply to the lactic system. The coenzyme functions as a highly specific carrier between lactic acid and the oxygen transporter.

The coenzyme of the hexosemonophosphate dehydrogenase cannot replace the lactic coenzyme in the lactic system.

The enzyme preparation may be purified extensively by treatment with kieselguhr and kaolin. Water-clear solutions can be prepared with Q_{O_2} values from 400 to 500.

The lactic enzyme is widely distributed in animal tissues. Its quantitative estimation has been carried out in the tissues of pigeon, rabbit and rat.

A new classification of dehydrogenase systems is presented based on recent research.

It is a pleasure to thank Mr Stanley Williamson for his invaluable assistance with many of the preparations. We are very grateful to Priv.-Doz. Dr Wagner-Jauregg for his gift of pure lactoflavin.

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CCXI. INVESTIGATIONS ON THE AMINO-ACIDS OF PLANTS.

I. TRYPTOPHAN CONTENT OF LEGUMINOUS PLANTS AT DIFFERENT STAGES OF GROWTH.

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During recent years we have followed the changes in the content of certain amino-acids in plants at different stages of growth. The objects of this work were firstly to obtain some information concerning the mechanism of amino-acid synthesis in plants and secondly to determine the physiological value of the nitrogenous constituents of plants. The nature of the work made it necessary to determine the entire amount of a given amino-acid present in the plant at any one time, whether free or as a component of peptides or different proteins.

In this respect our work differs essentially from the investigations carried out by Osborne *et al.* [1920; 1921], and particularly by Chibnall *et al.* [1923; 1933], Miller [1935; 1936], Morris [1934] and Kiesel *et al.* [1934] who examined the composition of the protein material which they isolated from the plant by various methods. Chibnall *et al.* determined the composition of the proteins of numerous plant species, employing very mild extraction whereby a maximum of 30 % of the plant protein could be isolated and assumed that the composition of the remaining 70 % was identical with that of the extracted protein. Morris, employing an efficient alkaline extraction, isolated, e.g. from hay and straw, the bulk of the protein present, and determined its composition. Whether some of the amino-acids are destroyed during the alkaline extraction seems to have remained unexamined. Neither Osborne, Chibnall nor Morris considered the possibility that the composition of the plant protein might vary during different stages of growth.

Kiesel *et al.*, working with Chibnall's method of extraction, have determined the composition of the protein material extracted from various plants at different stages of growth. They found that, for instance, the content of histidine and proline in the leaves of *Citrullus edulis* varied with the stage of growth, whilst that of the other amino-acids examined was fairly constant. It is not clear whether these variations were due to variations in the composition of a given protein, or whether the extracts in the different cases contained varying amounts of different proteins. Neither can any estimate of the total amounts of the different amino-acids present in the plant be obtained from Kiesel's data. The only work known to us, in which the total content of an amino-acid in the plant has been quantitatively determined at different stages of growth, is that of Klein & Tauböck [1932] on arginine. The analytical data presented in their paper show that the arginine content increased during the later stages of growth. Kretz's [1922] microscopical observations indicate that the meristem of the growing plant contains much tryptophan, whereas little is found in other tissues.

Our observations so far have thrown some light on the variations of aspartic acid and tryptophan in plants at different stages of growth [Virtanen & Laine, 1935]. The present paper is a report of our work on tryptophan and of the experimental technique employed.

EXPERIMENTAL.

In determining the tryptophan content of green plants the following considerations should be borne in mind: (1) the method of hydrolysis, and (2) the clarification of the extract for its colorimetric examination. In regard to the hydrolysis of the plant material, we found that Deseö's [1934] method, originally employed for pure proteins, was well suited for the estimation of tryptophan. In this method the material is hydrolysed in the autoclave with 20 % NaOH under 2–8 atmospheres' pressure. According to our experience this procedure causes no decomposition of tryptophan.

Before the colorimetric analysis the extract must be freed from colouring matter and carbohydrates. This is best done according to Folin & Marenzi [1929]. The last traces of colouring matter were removed by precipitation with lead acetate. The tryptophan content of the clear extract was then determined by the colorimetric method of Winkler [1934].

A weighed amount of the minced fresh material, containing 1–10 g. dry matter (or a corresponding quantity of dried plant material), is placed in a 100 ml. Erlenmeyer flask, together with 50 ml. of 20 % NaOH. The neck of the flask is wrapped in vegetable parchment, whereupon the flask is heated in the autoclave for 2 hours under 5 atmospheres' pressure (157°). Immediately after autoclaving, 75 ml. of 7 N H_2SO_4 and 25 ml. of saturated Ag_2SO_4 solution are added to remove Cl^-, followed by 5 g. of kaolin. The mixture is stirred and centrifuged. The residue is washed at the centrifuge with 100 ml. of 5 % H_2SO_4 and the combined solutions are treated with 25 ml. of 10 % $HgSO_4$ in 5 % H_2SO_4. Upon standing overnight, tryptophan precipitates as the Hg compound. The precipitate is centrifuged and washed once at the centrifuge with 100 ml. of 0·1 N H_2SO_4. The washed precipitate is suspended in 50 ml. of 0·1 N H_2SO_4, heated gently and decomposed with H_2S. The HgS is removed by filtration and washed with water, and the filtrate is freed from H_2S with a stream of CO_2. The solution is then clarified with 250 mg. of solid lead acetate and the precipitate is filtered and washed with water. The filtrate is transferred into a 100 ml. volumetric flask and made up to mark with distilled water. The tryptophan content of the solution is determined in aliquots of 1 ml. The standard tryptophan solution is prepared by treating 1 g. of Hammarsten-caseinogen in exactly the same manner. The tryptophan content of caseinogen, obtained by this procedure, is 1·20 % (against pure tryptophan standard).

1 ml. of the solution to be examined is measured into a test-tube and 1 ml. of the caseinogen standard into another tube; to both test-tubes are then added 3 ml. of a solution containing 0·2 ml. of glyoxylic acid solution (according to Benedict), 0·3 ml. of $M/100$ $CuSO_4$ and 2·5 ml. of distilled water. The tubes are cooled in an acetone-ice mixture, together with the H_2SO_4 to be employed. 5 ml. of cold conc. H_2SO_4 are then slowly added to the test-tubes, care being taken to avoid a rise of the temperature of the mixture. The tubes are next shaken and kept at room temperature for 3 hours or preferably overnight, whereupon they are heated in the water-bath for 5 min. After cooling the solutions are examined

colorimetrically and the tryptophan content of the extract is calculated on the basis of the caseinogen standard.

We made several control experiments to check the accuracy of the above method. In these experiments varying amounts of pure tryptophan were added to the plant material. The following data will illustrate the range of error involved. The plant material employed was dried clover, ground to a fine powder.

Exp.		Tryptophan found, mg.	Recovery of added tryptophan, %
1	2 g. powdered clover	9·8	--
2	2 g. powdered clover + 5 mg. tryptophan	14·6	96
3	2 g. powdered clover + 10 mg. tryptophan	19·7	99
4	2 g. powdered clover + 50 mg. caseinogen*	16·2	107

* Contained 6·0 mg. tryptophan.

The results show that the added tryptophan is satisfactorily recovered by the above method.

The tryptophan content of peas at different stages of growth.

On 27 March 1935, 12 pea seeds of equal size were planted in 20 earthenware jars, filled with quartz sand. The seeds were inoculated with the specific nodule organism. The *p*H of the sand was maintained at 6·5. The plants were watered with N-free Hiltner's solution. No artificial illumination was employed. The plants grew in the greenhouse uniformly and rapidly. For each analysis 20 plants of equal size were taken. The tryptophan determinations were made on two lots of 5 plants each. All colorimetric determinations were made in duplicate. The remaining 10 plants were used in lots of 5 plants each for the determination of dry matter and total N (Kjeldahl). The last plants were harvested on 6 June. The results are given in Table I and Fig. 1.

Table I.

Time of growth days	Dry wt. of 5 plants g.		N in 5 plants mg.		Tryptophan in 5 plants mg.		Tryptophan N % of total N	Remarks
0	(*a*) 1·073 (*b*) 1·075	1·074	(*a*) 42·8 (*b*) 43·5	43·2	(*a*) 3·36 (*b*) 3·29	3·33	1·06	Seeds
12	(*a*) 0·994 (*b*) 0·995	0·995	(*a*) 40·0 (*b*) 47·5	43·8	(*a*) 3·33 (*b*) 3·52	3·43	1·07	
16	(*a*) 1·100 (*b*) 1·100	1·100	(*a*) 46·62 (*b*) —	46·6	(*a*) 4·00 (*b*) 4·58	4·29	1·26	
20	(*a*) 1·398 (*b*) 1·423	1·411	(*a*) 58·38 (*b*) 62·02	60·2	(*a*) 9·00 (*b*) 9·67	9·34	2·13	
28	(*a*) 2·313 (*b*) 2·455	2·384	(*a*) 75·04 (*b*) 80·92	78·0	(*a*) 6·60 (*b*) 6·80	6·70	1·18	Start of flowering
34	(*a*) 2·528 (*b*) 2·868	2·698	(*a*) 83·16 (*b*) 88·08	85·6	(*a*) 7·00 (*b*) 7·80	7·40	1·18	
42	(*a*) 3·152 (*b*) 3·882	3·517	(*a*) 100·9 (*b*) 114·0	107·5	(*a*) 8·30 (*b*) 10·70	9·50	1·21	
71	(*a*) 9·365 (*b*) 8·640	9·002	(*a*) 399·4 (*b*) 421·3	410·4	(*a*) 37·5 (*b*) 42·8	40·4	1·32	Fully ripe
71	3·257		203·3		17·1		—	Pods
	5·565		207·5		20·3		—	Vines

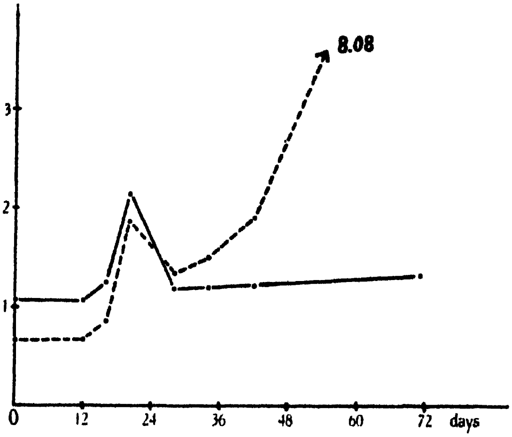

Fig. 1. •——• Tryptophan-N as % of total N. •- - - -• Tryptophan, mg. per plant.

The tryptophan content of red clover at different stages of growth.

The material was obtained from a clover field in the vicinity of Helsinki. All foreign material was carefully removed from the samples, whereupon these were thoroughly minced. Part of the minced material was used for the determination of dry matter and of carotene. The carotene determinations were made on duplicate samples of 40 g. each according to Willstätter. The remaining material was dried *in vacuo* at 100° for 5 hours, and ground to a fine powder. Tryptophan was determined on duplicate samples of 1 g. of the dry powder. The results are shown in Table II.

Table II.

Date of sampling	Length of clover cm.	Dry matter %		Total N mg. per g. dry matter		Tryptophan mg. per g. dry matter		Tryptophan-N % of total N	Carotene mg. per g. dry matter		Carotene % of dry matter
June 6	20	(*a*) 12·30 (*b*) 12·42	12·36	(*a*) 40·88 (*b*) 41·16	41·02	(*a*) 3·81 (*b*) 3·70	3·76	1·25	(*a*) 0·141 (*b*) 0·137	0·139	0·0139
„ 12	25	(*a*) 12·90 (*b*) 12·58	12·74	(*a*) 37·52 (*b*) 37·80	37·66	(*a*) 3·97 (*b*) 3·85	3·91	1·42	(*a*) 0·153 (*b*) 0·130	0·142	0·0142
„ 17	30	(*a*) 11·40 (*b*) 11·48	11·44	(*a*) 35·56 (*b*) 35·56	35·56	(*a*) 4·76 (*b*) 5·04	4·90	1·90	(*a*) 0·144 (*b*) 0·146	0·145	0·0145
„ 21	35	(*a*) 11·0 (*b*) 10·98	10·99	(*a*) 30·80 (*b*) 31·08	30·94	(*a*) 5·60 (*b*) 5·71	5·66	2·50	(*a*) 0·182 (*b*) 0·182	0·182	0·0182
„ 27	55	(*a*) 11·10 (*b*) 11·30	11·20	(*a*) 26·88 (*b*) 27·16	27·02	(*a*) 3·66 (*b*) 4·10	3·88	1·97	(*a*) 0·131 (*b*) —	0·131	0·0131
July 4*	75	(*a*) 12·89 (*b*) 13·0	12·95	(*a*) 23·94 (*b*) 24·22	24·08	(*a*) 2·57 (*b*) 2·75	2·66	1·51	(*a*) 0·059 (*b*) 0·067	0·058	0·0058
„ 8	80	(*a*) 17·95 (*b*) 17·95	17·95	(*a*) 22·12 (*b*) 22·26	22·19	(*a*) 2·38 (*b*) 2·42	2·40	1·46	(*a*) 0·051 (*b*) 0·049	0·050	0·0050
„ 20	80	(*a*) 18·24 (*b*) 18·30	18·27	(*a*) 22·26 (*b*) 22·40	22·33	(*a*) 2·16 (*b*) 2·37	2·27	1·40	(*a*) 0·057 (*b*) 0·067	0·062	0·0062
Aug. 6	80	(*a*) 23·90 (*b*) 24·10	24·00	(*a*) 18·34 (*b*) 18·62	18·48	(*a*) 1·99 (*b*) 2·11	2·05	1·51	(*a*) 0·040 (*b*) 0·040	0·040	0·0040

* Start of flowering.

CONCLUSIONS.

The variations in the tryptophan content are, on the whole, very similar both with peas and with clover. It is particularly interesting to note that in both experiments the % tryptophan content (total N basis) reaches a maximum at an early stage of growth, before blooming. At this stage the % of tryptophan-N in both plant species is double that noted in the seeds. The % content of tryptophan then falls rapidly so that, at the start of blooming, it is but slightly higher than in the seeds. There is very little change during the subsequent stages of growth, excepting a slight rise at about the time the seeds begin to ripen.

The experiments with peas, in which the total tryptophan content of 5 plants was determined at various stages of development, show that the above fall of the tryptophan-N : total N ratio was not exclusively due to a rise in the total N, but that the absolute content of tryptophan has also fallen (from 9·3 to 6·7 mg. per 5 plants). This is a most interesting result, showing that the lowering of the tryptophan content at the later stages of development cannot be explained by assuming that amino-acids other than tryptophan are formed in larger quantities at about the start of blooming, but that part of the tryptophan actually disappears from the plant at this stage. It is also interesting to note that during the earliest stages of growth about one-half of the rise in the total N is ascribable to the increase in tryptophan-N, although the latter constitutes only about 1–2 % of the total N. This remarkable rise in the tryptophan content during the early stages suggests that tryptophan plays an important part in the metabolism of the immature plant, possibly as a source of growth-promoting factors (β-indolylacetic acid).

It is also seen that the carotene content of clover reaches a maximum simultaneously with the tryptophan content. Virtanen *et al.* [1933] have already shown that in the green plant the contents of carotene and of vitamin C are highest during the period of most vigorous growth.

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CCXII. THE ASSAY OF *TRANS*DEHYDROANDROSTERONE AND ITS EFFECTS ON MALE AND FEMALE GONADECTOMIZED RATS.

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In our previous papers [Korenchevsky, 1935–36; Korenchevsky *et al.* 1934–36] we have studied the effects of those sexual hormones which are known to be present in the organs or excreted in the urine, namely, androsterone, testosterone and oestrone.

In the present paper the series of male hormones is concluded by giving the results of our experiments with *trans*dehydroandrosterone. It has not yet been proved in the case of human urine that the oestrogenic hormone in the urine of the male is oestrone nor has it been proved for any species that the male activity of female urine is due to androsterone, *trans*dehydroandrosterone or testosterone. Häussler [1934] and Girard (personal communication), however, isolated a chemically pure preparation of oestrone from the urine of stallions. It is therefore almost certain that the oestrogenic activity of human urine and that of other mammals is also due to oestrone. In addition to oestrone Girard (personal communication) isolated from the urine of stallions compounds similar to, but in some respects different from, equilenin. We have therefore studied the effect of oestrone on males and, since at least some of the male hormones mentioned above are suspected of being the cause of the specific sexual activity of the urine and blood of females, the effect of these male hormones on females was investigated.

Recently Koch [1936] made a careful quantitative examination of the male and female sexual activities in the human urine of both sexes.

A. *Previous experiments with* trans*dehydroandrosterone.*

*trans*Dehydroandrosterone (hereinafter referred to as T.D. androsterone) was isolated from male urine by Butenandt and his co-workers [1934; 1935]; by Schoeller *et al.* [1935] and by Oppenauer [1935], the quantity of this hormone in the urine being about the same as that of androsterone. These workers, as well as Ruzicka & Wettstein [1935, 1, 2], Ruzicka *et al.* [1935] prepared this hormone artificially. The biological properties of this hormone have been studied very little and some of the results obtained are contradictory. While Butenandt found that 1 capon unit was contained in about 600γ, Tschopp [Ruzicka *et al.* 1935; Tschopp, 1936] stated that 1 C.U. was contained in about 150–200γ, but in another paper [Ruzicka & Wettstein, 1935, 1] gave the figure as 500γ. Deanesly & Parkes [1936, 1] found 1 C.U. in 300γ.

Tschopp [Ruzicka & Wettstein, 1935, 2] found that up to 500γ of T.D. androsterone injected daily for 10 days had no definite effect on the seminal vesicles and prostate of castrated rats. Deanesly & Parkes [1936, 1], also injecting for 10 days, found that doses of 1 and 2 mg. per day had a slight effect on these organs.

Butenandt *et al.* [1935] found no oestrogenic effect of 2 mg. of T.D. androsterone on the vagina of ovariectomized mice, but obtained positive results with normal infantile females. Tschopp [1936], working with ovariectomized rats, confirmed this statement by Butenandt of the lack of oestrogenic effect of T.D. androsterone. Deanesly & Parkes [1936, 2], in 4 of 5 rats, found, however, that total doses of 2–3 mg. had oestrogenic activity on the vagina in both normal and ovariectomized rats.

B. *Present experiments.*

The experiments were performed with T.D. androsterone artificially prepared by Ruzicka and his co-workers and kindly supplied by Messrs Ciba Ltd. The hormone was dissolved in sesame oil and was investigated by our usual technique which is fully described in our previous papers. The animals were injected in the assay experiments for 7 days and in the prolonged experiments for 21 days, the daily dose being injected in half-doses twice a day. The control rats, normal and gonadectomized, were injected with the sesame oil only.

I. *The assay of T.D. androsterone.*

Using Korenchevsky's method, the assay was performed on 38 rats belonging to 8 litters. The ages of the litters at dissection varied from 57 to 65 days. The results obtained are given in Table I. Although the effect on the sexual organs and thymus was clear, it was small even with such a large dose as 4 mg. per day. One rat unit was found to be contained in about 940γ, as measured by a 40% increase in the actual weight of the prostate.

Table I. *Assay experiment.*

The effect of 7 days' injection of testosterone on sexual organs, thymus and body weight of male castrated rats.

Organ	Weights	Control rats	Rats injected with (mg.)			
			0·8	1	2	4
		A. Average weights.				
Seminal vesicles (mg.)	Actual	9·4	15·1	15·4	18·0	25·7
Prostate (mg.)	Actual	54	72	77	85	104
Prostate with seminal vesicle (mg.)	Actual	63	87	92	103	130
Penis (mg.)	Actual	72	94	92	104	120
Preputial glands (mg.)	Actual	60	95	110	116	118
Thymus (mg.)	Actual	666	658	608	599	568
Final body weight (g.)	Actual	211	222	228	227	219
No. of rats in each group	—	7	9	9	7	6
		B. Percentage changes in weight.				
Seminal vesicles	Actual	—	+61	+64	+92	+173
	Per unit of body weight	—	+53	+52	+79	+164
Prostate	Actual	—	+33	+43	+57	+ 93
	Per unit of body weight	—	+27	+33	+47	+ 86
Prostate with seminal vesicles	Actual	—	+38	+46	+64	+106
	Per unit of body weight	—	+30	+35	+52	+ 98
Penis	Actual	—	+31	+28	+44	+ 67
	Per unit of body weight	—	+25	+19	+35	+ 62
Preputial glands	Actual	—	+58	+83	+93	+ 97
	Per unit of body weight	—	+49	+70	+79	+ 90

II. *Prolonged injection of T.D. androsterone, alone or simultaneously with oestrone, into castrated male rats.*

Experiments were performed on 42 rats belonging to 9 litters, half the litters being killed at about 68 days old and the rest at about 86 days old. Therefore, in Table II, in which all the other details are also summarized, the data for these two age groups are given separately. As no normal control rats were available for the age group 86 days, the average weights of the organs of 5 normal rats (about 96 days old) from another experiment were used for comparison and inserted into the corresponding group of col. i, Table II.

Table II. *Effect of prolonged injection of sexual hormones on male rats.*

The average actual weights of the organs of castrated rats injected with sexual hormones as compared with those of the organs of normal or castrated control rats injected with oil only.

	i	ii	iii	iv	v	vi	vii
			Castrated rats injected with				
	Normal control rats	Castrated control rats	T.D. androsterone			Oestrone 6γ + T.D. andro-sterone	Av. age of rats
Organ			1 mg.	2 mg.	3 mg.	1 mg.	days
Seminal vesicles (mg.)	677	11	18	—	29	23	68
	999	14	18	31	—	—	86
Prostate (mg.)	799	57	81	—	105	97	68
	1250	64	91	127	—	—	86
Seminal vesicles + prostate (mg.)	1476	68	99	—	134	120	68
	2249	78	109	158	—	—	86
Penis (mg.)	280	63	98	—	120	110	68
	328	85	110	132	—	—	86
Preputial glands (mg.)	137	49	101	—	160	124	68
	144	57	117	109	—	—	86
Adrenals (mg.)	56	79	63	—	55	64	68
	60	85	71	66	—	—	86
Hypophysis (mg.)	9·2	13·3	12·5	—	12·7	14·3	68
	10·8	14·5	14·7	14·5	—	—	86
Thymus (mg.)	462	579	584	—	549	504	68
	315	615	543	579	—	—	86
Liver (g.)	13·3	10·9	11·0	—	11·7	9·4	68
	13·6	11·6	12·6	13·4	—	—	86
Kidney (g.)	2·06	1·65	1·76	—	1·72	1·72	68
	2·31	1·91	1·93	1·96	—	—	86
Heart (mg.)	928	703	780	—	772	706	68
	960	931	919	973	—	—	86
Average final body weight (g.)	295	244	250	—	255	220	68
	367	314	319	327	—	—	86
No. of rats in group	2(+5)	9	14	7	2	8	—

In order to economize space, the weights of the organs per unit of body weight are not given, but will be mentioned in the text in those cases where the character of the changes, as judged by these two data, differs.

Sexual organs and thymus. In spite of the fact that in this prolonged experiment the period of injection of T.D. androsterone was three times longer than in the assay experiment, the hypertrophy of the sexual organs and the rate of involution of the thymus (Table II, cols. iii, iv, v) were only slightly greater than those observed in the assay experiments (Table I).

The addition of oestrone (Table II, col. vi) caused only a slight increase in the effect of the similar dose (col. iii) of T.D. androsterone.

Adrenals and liver. In contrast to the effects described above, the injections brought about a return to the normal weight in the case of the hypertrophied "castration" adrenals and the decreased "castration" liver, the effect increasing with the larger doses. Whilst the addition of oestrone did not definitely influence the changes in weight produced in the adrenals, it appeared to prevent the hypertrophy of the liver, even in some cases causing a decrease in the actual weight. The data per unit of body weight however show that this failure of the liver to hypertrophy must be partly explained by the smaller liver observed in animals with a smaller body weight, the smaller body weight in this case being caused by the injection of oestrone.

Other organs, fat deposition and body weight. Castration produces hypertrophy of the hypophysis (col. ii) and a slight decrease in the weight of the kidneys and heart (col. ii). With perhaps the exception of some effect on the heart, the injections did not bring about a return to the normal weight in the case of these organs. The changes in the weight of the heart however were very small and are only mentioned because they are in agreement with the results obtained with the other male hormones [Korenchevsky *et al.* 1935; 1936].

No changes were observed in the thyroids, spleen, fat deposition and body weight after the injections; excepting in the rats injected simultaneously with oestrone and T.D. androsterone, in which the body weight, gain in body weight and fat deposition were less than in the rats injected with T.D. androsterone alone. This is a typical effect of oestrone as was shown in our previous papers.

III. *Prolonged injections of T.D. androsterone, alone or simultaneously with oestrone into female spayed rats.*

The experiment was performed on 61 rats belonging to 13 litters, ovariectomized before sexual maturity and killed at ages varying from 61 to 77 days. The injections were begun from 14 to 34 days after ovariectomy and continued daily for 21 days. The data obtained and other details of the experiments are summarized in Table III. In the normal females the weights of the uteri of only those rats which were in dioestrus were used for the average.

The sexual organs. While not large, the stimulating effect of T.D. androsterone on the uterus and vagina was quite definite (cols. iv, v, vi), though less than that of oestrone (col. iii). Only in some cases was the addition of oestrone (cols. vii-ix) followed by a slight increase in the degree of hypertrophy observed with oestrone alone (col. iii).

In contrast to this small effect of T.D. androsterone on the uterus and vagina is the high degree of hypertrophy of the female preputial glands which exceeded even the normal size whilst the addition of oestrone did not have any effect on the weight of these glands.

Thymus. The rate of involution of the thymus was definitely increased by the injection of T.D. androsterone but was less than in the rats injected with oestrone alone or simultaneously with T.D. androsterone.

Adrenals and liver. As in castrated males, injections of T.D. androsterone (cols. iv–vi) were followed by a decrease in the weight of the adrenals and an increase in the weight of the liver. When oestrone was injected simultaneously these effects decreased in degree or disappeared entirely.

Table III. *Effect of prolonged injection of sexual hormones on female rats.*

The average actual weights of the organs of spayed rats injected with sexual hormones as compared with those of the organs of normal or spayed control rats injected with oil only.

	i	ii	iii	iv	v	vi	vii	viii	ix
			Spayed rats injected with						
Organ	Normal control rats	Spayed control rats	Oestrone 6γ	T.D. androsterone 1 mg.	T.D. androsterone 2 mg.	T.D. androsterone 4 mg.	Oestrone 6γ + T.D. androsterone 1 mg.	Oestrone 6γ + T.D. androsterone 2 mg.	Oestrone 6γ + T.D. androsterone 4 mg.
Uterus (mg.) (dioestrus)	359	33	123	56	57	69	141	140	110
Cervix of uterus (mg.)	103	13	50	22	22	30	57	53	47
Vagina (mg.)	241	130	186	143	152	163	206	200	204
Female preputial glands (mg.)	116	76	78	190	176	146	141	158	216
Adrenals (mg.)	62	80	79	74	62	55	71	73	70
Thyroids (mg.)	16	19	18	21	22	22	20	18	18
Hypophysis (mg.)	13·3	12·3	12·9	12·2	12·2	11·8	13·6	12·4	12·1
Thymus (mg.)	413	687	499	519	564	549	477	465	471
Liver (g.)	8·2	9·0	7·4	10·0	10·5	11·1	8·5	8·6	9·8
Kidney (g.)	1·40	1·59	1·53	1·53	1·56	1·54	1·59	1·51	1·52
Heart (mg.)	628	694	619	677	687	713	635	644	647
Fat (g.)	7	7	6	7	7	8	5	5	6
Average final body weight (g.)	181	218	189	213	208	210	191	182	206
Gain in body weight (g.)	59	89	40	85	71	82	51	33	56
No. of rats in group	8	13	11	4	6	4	5	6	4

Thyroids. When calculated per unit of body weight, no increase in the weight of the thyroids was seen after ovariectomy, whilst after T.D. androsterone injections there was a slight increase both in the actual weight and in the weight per unit of body weight of these glands. The increase however was small and therefore until the histological examination is completed the significance of this increase cannot be ascertained.

Other organs, fat deposition and body weight. No definite changes in the weight of the hypophysis, kidneys and heart or in the fat deposition and body weight were observed after the injection of T.D. androsterone, whilst injections of oestrone alone (col. iii) or simultaneously with T.D. androsterone (cols. vii–ix) caused a decrease in the final body weight, the gain in body weight and the fat deposition as compared with spayed control rats (col. ii).

DISCUSSION.

Before the histological investigation is completed, it is not possible to make a final evaluation of the results obtained, more especially as regards the question whether the return to or towards the normal weight is accompanied by the restoration of the normal histological picture.

Several points however are clearly demonstrated in the results obtained.

Male rats.

The rat unit. The rat unit (as defined by Korenchevsky) is contained in 8γ of testosterone, 21γ of androsteronediol, 170γ androsterone and 940γ of *trans*dehydroandrosterone. Thus from the point of view of the smallest active dose T.D. androsterone is a very weak sexual hormone.

The qualitative effect on the sexual organs. The prolonged experiments confirm the low activity of this hormone on the sexual organs. It was shown in our previous paper (see summarizing Table VII, Korenchevsky *et al.* 1936, p. 572) that the ratio (× 100) of the percentage increase in the weight of the prostate to that of the seminal vesicles observed after injections of the hormone indicates how nearly the changes approximate to the normal development of the sexual organs. To show this, a comparison is made with the ratio obtained during the normal growth of the organs in normal rats and after the injection of gonadotropic hormone into normal infantile rats.

It is seen from Table IV that in the short-period injections (cols. i, ii) this ratio for T.D. androsterone was on the average 59 (col. ii), which figure is close to those obtained with androsteronediol (64) and testosterone (51), but considerably different from that obtained with androsterone (106). The normal ratio for comparison in these short-duration experiments is given by the gonadotropic hormone which in a few days forces the development of the prostate and seminal vesicles of normal infantile animals. This ratio is 54 (col. ii). In short-duration experiments therefore T.D. androsterone as well as androsteronediol and testosterone (but not androsterone) give a ratio approximating to the normal.

Table IV. *Ratio (× 100) of percentage increase in actual weight of prostate to that of seminal vesicles after injection of* trans*dehydroandrosterone compared with this ratio obtained with other hormones or observed during natural growth of the organs in normal rats.*

	i	ii	iii	iv
	Short-period injections		Long-period injections	
	Daily dose γ	Ratio prostate to seminal vesicle	Daily dose γ	Ratio prostate to seminal vesicle
*trans*Dehydroandrosterone	800	54	1000	70
	1000	67	2000	81
	2000	62	3000	51
	4000	54	—	- -
*trans*Dehydroandrosterone	Average	59	1000–2000	76
	-	—	3000	51
Androsterone	Average	106	450–2200	126
	—	—	3600 or more	55
Androsteronediol	Average	64	175–350	54
	---	—	700 or more	24
Testosterone	Average	51	33–167	37
	—	—	500–1000	29
Gonadotropic hormone	Average	54	—	—
Normal development in normal rats	—	—	—	25

For comparison of the results of the long-period injections (cols. iii, iv) the normal ratio should be taken as that which is obtained from the normal development of the prostate and seminal vesicles in normal rats (=25, see col. iv).

Usually injections of the higher doses of the hormones give a closer approximation to this normal standard [Korenchevsky *et al.* 1936]. It can be seen from Table IV (cols. iii, iv) that this ratio for the high doses of T.D. androsterone (51) and androsterone (55) is very different from the normal ratio (25), whilst for androsteronediol (24) and for testosterone (29) it approximates closely to the normal ratio. In general, however, taking into consideration both the short and long experiments, the development of the prostate and seminal vesicles obtained is qualitatively closer to the normal with T.D. androsterone than with androsterone, though this latter hormone is quantitatively the more potent of the two.

Although even the largest doses of T.D. androsterone used did not bring about any considerable increase in the weights of the seminal vesicles, prostate or penis, the weight of the preputial glands became normal or nearly normal.

The effect on other organs. The weights of the thymus, adrenals, liver, kidneys and heart, which change after castration, were restored to or towards the normal weight by androsterone, by androsteronediol and by testosterone. The decreased gain in body weight of castrated rats was also improved by each of these three hormones [Korenchevsky *et al.* 1935; 1936]. T.D. androsterone produced a similar effect in the case of the adrenals and liver, but the action on the thymus and heart was much weaker and in some cases absent altogether. The weight of the kidneys and the gain in body weight also showed no definite change with T.D. androsterone.

Female rats.

The sexual organs. The effect of T.D. androsterone on the uterus and vagina was greater than that of androsterone [Korenchevsky *et al.* 1935, Table V, p. 2543], but less than that of androsteronediol (*ibid.*) or of testosterone [Korenchevsky *et al.* 1936, p. 567]. Each of the four hormones brought about hypertrophy of the female preputial glands to a weight and size greater than normal. All these four male hormones, therefore, undoubtedly have some of the properties typical of the female hormones.

The possible significance of the preputial glands in a differential assay of the male and female hormones. In spite of the sensitivity to male hormones of the preputial glands of both sexes, they are not suitable for the assay of male hormones since the variation in the weight of these glands both in uninjected and in injected rats is high, and can only be compensated by using a large number of animals. However, since the weight of these glands in both sexes is unaffected by oestrone or oestradiol [Korenchevsky *et al.* 1936], this difference may be helpful as an extra indication of the degree or absence of contamination of the female hormone preparations with male hormones.

Adrenals. Owing to the greater irregularity in the weights of the adrenals in female than in male rats, we are still unable to say definitely how frequently after ovariectomy these glands hypertrophy. While we intend in our next experiments to collect more data and to investigate this question histologically, at present we can only say that in our experiments ovariectomy was often followed by an increase in the weight of the adrenals, as can be seen, for example, in Table III (cols. i, ii). The injection of T.D. androsterone (cols. iv–vi) decreased this hypertrophy and with the large dose the normal weight was restored. This decrease in the size and weight of the adrenals was also seen after the injection of androsterone, of androsteronediol and (taking into consideration our hitherto unpublished results with testosterone) of testosterone. With respect to this effect of testosterone, the statement in our previous paper as to the absence of effect of this hormone on the weight of the adrenals [Korenchevsky *et al.* 1936, p. 573] made provisionally (as only a few rats were used) must be corrected. Thus, in both gonadectomized males and females, the weight and size of the adrenals are decreased by male hormones.

Liver. In both our published [Korenchevsky *et al.* 1936] and unpublished experiments with testosterone, no effect was seen on the weight of the liver, while with androsterone, androsteronediol and with T.D. androsterone (present experiments) there was a marked increase in the weight of the liver of ovariectomized rats.

Other organs and gain in weight. Except for a slight increase in the rate of involution of the thymus no definite influence of T.D. androsterone was observed on the other organs, fat deposition or gain in body weight. In contradistinction to this, androsterone, androsteronediol and testosterone caused a greater increase in the rate of involution of the thymus, slightly decreased the weight of the hypophysis and (androsterone and androsteronediol) increased the weight of the kidneys and gain in body weight [Korenchevsky *et al.* 1935; 1936].

The co-operative activity of T.D. androsterone and oestrone on males and females. In the case of males this activity was found to be very slight and in the females, unless shown on histological investigation, is doubtful. With androsterone, androsteronediol or testosterone on the other hand this co-operation with oestrone was definite [Korenchevsky *et al.* 1935: 1936].

SUMMARY.

1. Experiments were performed on 7 normal and 78 castrated males and 8 normal and 61 ovariectomized females, in order to investigate the effect on the weight of various organs of Ruzicka's artificial *trans*dehydroandrosterone, when injected alone or simultaneously with oestrone.

2. Korenchevsky's rat unit of T.D. androsterone was found to be contained in about 940γ.

3. The restorative effect of T.D. androsterone on the atrophied sexual organs of male and female gonadectomized rats was definite, but (except in the case of the preputial glands) small. The co-operative effect with oestrone was only slight (males) or doubtful (females).

4. Thus T.D. androsterone showed some of the properties typical of the female hormones.

5. The response of the preputial glands in both sexes to all the male hormones (so far investigated by us) and the lack of such a response to oestrone or oestradiol can be used as an additional check in the differential assay of male and female sexual hormone preparations.

6. T.D. androsterone caused a decrease in the weight of the adrenals, slightly increased the rate of involution of the thymus and increased the weight of the liver in both male and female gonadectomized rats.

7. The similarities and differences in the biological properties of T.D. androsterone and the other male hormones as investigated by us were discussed.

8. The ratio of the percentage increase in the weight of the prostate to that of the seminal vesicles after the injection of T.D. androsterone was found to be similar to that obtained with gonadotropic hormone, but different from that observed during the normal development of normal rats.

Grants from the Medical Research Council and from the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish to express our gratitude to Prof. A. Girard for his valuable information and for kindly supplying us with oestrone; and to Messrs Ciba Ltd., in particular to Dr K. Miescher, for the generous supply of *trans*dehydroandrosterone.

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CCXIII. ULTRAFILTRATION AND CONCENTRATION BY ULTRAFILTRATION BY A CENTRIFUGE METHOD.

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"ULTRAFILTRATION is an exceedingly slow and tedious operation and at present restricted to the laboratory." This statement, in a recent text-book of colloid chemistry [Thomas, 1934], will be more or less endorsed by most experimental workers, especially by those who only use this method occasionally. Concentration by ultrafiltration might seem a choice method for colloids which are inactivated easily, but so far it has only been successful in few cases. The ineffectiveness of this technique is principally due to clogging of the ultrafilter membrane by a deposit of the disperse phase; this reduces the filtration velocity very much or stops the process altogether. In many methods a scraping device is incorporated, but its advantage is doubtful, since a very thin deposit is as effective in retarding ultrafiltration as a thick one, and the scraper or stirrer cannot effectually remove it. Hence as a rule a small amount of ultrafiltrate is obtained in the first hours and after that not much more. Of course, an experienced worker can make membranes, which combine impermeability to protein with rapid ultrafiltration in the first hours, but for clinical application, especially, there is need of a method for collecting about 3 ml. of protein-free ultrafiltrate from about 10 ml. of serum or blood in half an hour's time, without requiring skill in the preparation of suitable membranes. Such a method is described in this paper.

The pressure necessary for rapid ultrafiltration is obtained by centrifugal force, as in De Waard's [1918] method. In this latter method however the filtering membrane is, as in all other procedures, at right angles to the direction of the filtering solutions, so that the membrane is clogged by the deposit in about 1 hour.

If however one uses a small cylindrical unglazed porcelain pot, covered on the inside by an ultrafilter membrane, the centrifugal pressure is exerted on the sidewall and bottom, whilst any peripheral part of the liquid which becomes increased in specific gravity is centrifuged towards the bottom. In this way clogging is prevented, since any part which becomes denser through ultrafiltration moves at right angles to the direction of the ultrafiltering stream.

The velocity of ultrafiltration now only depends on the centrifugal pressure, and the dimensions of the column of solution. The following example illustrates the situation: an ultrafilter tube is filled with 10 ml. of 1 % haemoglobin solution and centrifuged for 10 min.; in this time 2·1 ml. of protein-free ultrafiltrate are formed; a second rotation for 10 min. yields 1·3 ml., a third yields 1·0 ml. and a fourth 0·8 ml. So in 40 min. 5·2 ml. of ultrafiltrate have been obtained from 10 ml. of haemoglobin solution, the concentration of which is now doubled.

If the amount of Hb solution in the cylinder is brought up to 10 ml. at the end of each 10 min. interval, the production of ultrafiltrate proceeds at a constant rate of 2 ml. per 10 min.

As regards coating the porcelain cylinder with an ultrafiltration layer, it was desirable that (*a*) the membrane should be impermeable to colloids like serum proteins or haemoglobin, without at the same time being too slowly permeable to water and crystalloid solutes, (*b*) the ultrafilter layer should be easy to make and reproducible, and (*c*) the durability of this layer should be as good as possible or that the layer might be quickly regenerated. After many trials we found these conditions to a certain extent fulfilled by covering unglazed "chamotte" cylinders (which have fine pores and a smooth inner surface) with a layer of sodium water-glass (30 %). Potassium water-glass cannot be used. The membrane is made as follows.

The dry porous cylinders are filled with the water-glass and left standing for some minutes; the water-glass is then poured out again and the cylinders are kept upside down till no more liquid drains off. They are then set aside for 30 min. at room temperature. After that, they are rinsed with tap water for 1 to 2 min. and tested with haemoglobin solution.

For a 10 ml. covered cylinder filled with 1 % haemoglobin solution and rotating in an ordinary centrifuge at 3000 r.p.m. the amount of ultrafiltrate in 10 min. should be 1–2 ml. If, at the first trial the amount of protein-free ultrafiltrate is too small, e.g. 0·5 ml., this is corrected by rinsing with tap water for 10–15 min. The silicate covering appears to swell a little under this treatment and the permeability increases. If the contact with streaming water is too long, the swelling may lead to a permeability for colloids; the filters have then to be dried for a longer time, e.g. overnight, and generally the impermeability for colloids is restored. If this is not so, one has to apply a new coating of water-glass.

We have found that some porous cylinders are not suited for these water-glass membranes. We use a type having a soft and smooth surface with very fine pores; it is not known by any special name, but may be obtained at low cost from Messrs Marius, Utrecht, Holland. The filter tubes will stand a long and intensive use; we have used them for 4–5 hours every day, for 2 weeks at a time, without a significant change in permeability. Sometimes a slight decrease was observed, which was overcome by a short rinsing with tap water.

When not in use the filters are kept in moist paper in a cool place. They may then dry a little too much and consequently have a lessened permeability; this is treated as described above. It is remarkable that no deposit at all becomes observable when the filters are used for concentration by ultrafiltration. The concentration of the disperse phase steadily increases, and so it is possible to divide 10 ml. of a 1 % haemoglobin solution into 5 ml. of a 2 % solution and 5 ml. of a protein-free ultrafiltrate in about half an hour.

If, during this process, the volume of the contents of the tube is kept constant every 10 min., by replacing the lost water by small rods of a suitable solid, inert material, a 10-fold concentration may be reached in 1 hour. We use for this purpose an artificial resin, but any other solid with a specific gravity just over 1 may be used.

When larger amounts of colloidal solution are to be concentrated, one can use 4 tubes at a time, keeping them filled every 10 min. and finally reducing the number of tubes to 3, 2 and 1 when the total volume is reduced to 10 ml. We have e.g. concentrated 200 ml. of urine, containing 0·04 % of Bence Jones protein to 4 ml. of a 2 % solution in 5 hours' time. It must be mentioned that the porous wall of the cylinder we used can take up about 2 ml. of solution, so that, if the ultrafiltrate is required for analyses, the first 2 ml. must be discarded. Owing to the rapid ultrafiltration process this takes 10–15 min., so that not much time is lost.

SUMMARY.

By coating porcelain cylinders with water-glass, ultrafilters are obtained which are very suitable for ultrafiltration and concentration by ultrafiltration by means of the centrifuge. The filters are not clogged by the disperse phase and will stand heavy use.

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CCXIV. COMPOUNDS OF SERUM PROTEINS WITH POLYSACCHARIDES.

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The proteins contain, apart from the CONH group, a large number of the most varied side-chains derived from amino-acid residues. According to Przyłęcki [1932] the side-chains form in proteins, together with the binding group CONH, fourteen typical groups. Thanks to the presence of these groups, the proteins possess affinities with a large number of the most varied substances, such as (1) compounds possessing polar groups (polysaccharides, fats, lecithins), (2) compounds possessing non-polar groups, such as: light petroleum or other hydrocarbons, benzene, toluene, cholesterol, etc. The presence or absence of certain groups, as also their heterogeneous mutual position contribute to cause the greatly differing behaviour of the various proteins. The investigations of Hardy [1905] and Pauli & Valkó [1933] furnish classical examples of the functional relation between the structure and the properties of proteins. Przyłęcki and his co-workers in a long series of papers [1931–34] have examined the relation between the composition of proteins and their ability to form compounds with lipoids, fats, nucleic acids and polysaccharides; special regard was paid to the dependence of binding on the presence and amount of amino-acids, and their distribution in the protein molecule. According to Przyłęcki [1932] it is necessary to distinguish two types of binding between proteins and other substances, viz.:

A. Complex compounds formed by the binding of polar groups of proteins with the polar groups of other substances free of ionized groups (Willstätter's symplexes).

B. Compounds of the multi-polar type, formed by the binding of ionized groups (e.g. H_3PO_4, amylopectin etc.) with the ionized groups contained in the protein similar to the salts.

In order to solve the problem of binding between proteins and various non-ionized groups, Przyłęcki *et al.* [1935; 1936] conducted two types of experiments.

I. Polysaccharides (phosphoric acid-free) were bound with pure amino-acids and with synthetic polypeptides. The formation of compounds between amylose and various amino-acids was examined by means of polarimetric, refractometric, cataphoretic, cryoscopic and ultrafiltration methods. The results attained indicated that the following amino-acids do not combine with amylose:

(*a*) glycine, alanine, valine, leucine;

(*b*) aspartic acid, glutamic acid, asparagine;

(*c*) cystine, phenylalanine, tryptophan, hydroxyproline, lysine and histidine;

(*d*) the CONH group (pentaglycylglycine, leucylglycylglycine, glycylglycine, glycylanhydride).

Amongst the amino-acids, the following are exceptions: tyrosine and arginine (with its guanidine group). Tyrosine, for instance, could bind as much as 20–40 % of the polysaccharide.

On the basis of these experiments, it was shown that of the large number of side-chains and the binding group (CONH) entering into the composition of the protein molecule, only arginine and tyrosine combine with polysaccharides. Experiments on free amino-acids cannot of course yield proof that only the above amino-acids can be taken into account in the binding of polysaccharides with proteins, since:

(1) Free amino-acids do not necessarily react in the same way as when forming part of a protein molecule.

(2) Not all the amino-acids entering into the composition of the protein molecule have been identified.

(3) Many proteins contain various prosthetic groups or contaminants.

For these reasons a second series of experiments was undertaken, a part of which is described herein, the rest having been given by Przyłęcki *et al.* [1936].

II. In most of the experiments the results with free amino-acids or with polypeptides were in agreement with those for the corresponding proteins. These experiments indicate that some proteins bind much amylose, whilst others bind or adsorb only insignificant amounts. The positive role of arginine and tyrosine was also proved, as only those types of protein bound amylose which contained large quantities of tyrosine (caseinogen 5·4 %, serum globulin 6·7 %, silk fibroin 11·0 %), or of arginine (clupein with 87 % arginine yielded as much as 70–90 % of amylose in the precipitate).

Of the serum proteins examined by Przyłęcki *et al.* [1931–36] globulin bound amylose most strongly, while albumins bound it only under certain given conditions. As the experiments on the combination of serum protein with amylose were conducted under various conditions and with various methods, using substances containing lipoids, it was sought to elucidate the following points:

(1) how far the various kinds of experimental methods used affect the binding;

(2) which of the globulins, whether eu- or pseudo-, has the greatest binding capacity;

(3) whether phosphorus-free amylose is also bound by serum proteins;

(4) whether the fat content of the serum proteins affects their ability to bind amylose.

EXPERIMENTAL.

Horse and ox serum proteins.

Separation was effected by fractional precipitation with $(NH_4)_2SO_4$, and purification by thrice-repeated solution and re-precipitation. The protein was then dialysed for 3 days against ordinary water and for 2 days against distilled water and then dried *in vacuo* over sulphuric acid at 35°.

Phosphorus-free amylose was prepared by electrodialysis for 12 hours of 2·5 % solution of rice starch in a Pauli electrodialyser at about 400 v. and 30–40 milliamps.; the solution was then evaporated at *p*H 7, to the desired concentration over an ordinary Bunsen burner. Phosphorus was in all cases found to be absent.

Fat-free protein was prepared by Young's method and in addition protein was used from which the fat was extracted by means of ether and methylene chloride. The fat contents of the proteins were determined by the method of Kumagayo-Suto.

Phosphoric acid in the amylose was determined by the help of ammonium molybdate after prior evaporation to dryness of the amylose with the addition of HNO_3.

2 % solutions or suspensions of proteins were used in the experiments, of which there were two series.

In the first series the protein was mixed with a 0·22 % solution of amylose, and after standing for 15 min. was precipitated by boiling or by changing the *p*H and then filtered out.

In the second series the protein was coagulated either by heating or by changing the *p*H before adding the amylose; 0·2 % (approx.) solution of amylose was added to a 2 % suspension of protein, brought to a suitable *p*H and the whole filtered after 15 min.

The combination between the protein and the amylose was determined by changing the concentration of amylose in the filtrate. The amylose in the filtrate was determined by the method of Pflüger. The tables give the percentage diminution in amylose concentration and the quantity of amylose bound by the protein in percentages of the latter.

In addition, experiments were conducted in the presence of neutral salts ($CaCl_2$, Na_2SO_4 and KCl).

RESULTS.

Protein coagulation and the binding of amylose by serum albumin.

In order to ascertain whether protein coagulated by boiling or by change of *p*H possesses the same ability to bind amylose as protein in the form of a sol, the following series of experiments was made:

(1) 50 ml. 2 % suspension of ox albumin + 50 ml. of 0·22 % solution of amylose + 0·5 g. of Na_2SO_4 (*p*H 4·0).

(2) 50 ml. 2 % suspension of ox albumin + 50 ml. of amylose + 0·5 g. $CaCl_2$ (*p*H 7·7).

(3) Same as No. 2, but *p*H 8·0.

(4) 50 ml. of ox albumin solution + 50 ml. amylose + 0·5 g. $CaCl_2$ (*p*H 7·7). Albumin precipitated by heating.

(5) Same as No. 4, but *p*H 8·0.

(6) 50 ml. of ox albumin solution + 50 ml. of amylose + 1 % Na_2SO_4 (*p*H 4·0).

(7) Same as No. 6, but albumin precipitated by heating, *p*H 8·0.

(8) 50 ml. of ox albumin solution + 50 ml. amylose (*p*H 6·17). The albumin solution before the addition of amylose was denatured without precipitation by heating after adding 5 ml. of *N*/10 NaOH and then precipitated by change of *p*H.

(9) Same as No. 8, but with horse albumin, *p*H 5·52.

It follows from the data in Table I that once albumin is coagulated it cannot bind amylose. If, however, the protein is in the form of a sol, a slight combination ensues. The addition of $CaCl_2$ does not specially affect the binding

Table I.

Exp. no.	*p*H	Salt added	Diminution in amylose concentration %	Amylose bound by protein (computed in % of protein)
1	4·0	0·5 % Na_2SO_4	0	6·0
2	7·7	0·5 % $CaCl_2$	0	0
3	8·0	0·5 % $CaCl_2$	0	0
4	7·7	0·5 % $CaCl_2$	21·5	2·25
5	7·7	0·5 % $CaCl_2$	25·5	2·2
6	4·0	0·5 % Na_2SO_4	0	0
7	8·0	2 % Na_2SO_4	29·2	3·4
8	6·17	0	28·9	2·6
9	5·52	0	35·1	4·8

between the albumin and the amylose as when using Na_2SO_4 we have very closely similar results. It is further seen that binding occurs only at moderately low *p*H; thus at *p*H 4 there is no binding, whereas at *p*H 5·52 binding occurs. If we denaturate the protein (without precipitating it by heating at a high *p*H), mix with amylose and then precipitate by change of *p*H, the figures obtained are practically the same as those for albumin solution precipitated by boiling (cf. Exp. 8 with Exps. 4 and 5). Horse albumin binds more strongly than ox albumin.

Fat content and the binding of amylose with pseudoglobulins.

In former researches, it was only found that globulins (both eu- and pseudo-) bind amylose strongly. In the present series of experiments it was sought to determine to which of the globulins this property should be ascribed, and whether the fat content affects binding. With these objects in view we took ordinary globulin and globulins defatted by the method of Young and by that of extraction with ether and methylene chloride. The following experiments were then carried out:

(1) 2 % suspensions of pseudoglobulin (horse) prepared by boiling in the presence of 2 % Na_2SO_4; 25 ml. suspension + 25 ml. amylose (*p*H 4·0).

(2) Same as No. 1, but *p*H 7·0.

(3) 2 % solution of pseudoglobulin (horse) dissolved at *p*H 8·5, amylose added and globulin precipitated at *p*H 5·0.

(4) 2 % solution of pseudoglobulin (defatted by extraction with ether and methylene chloride), dissolved in 4 % KCl, amylose added, and precipitated by boiling (*p*H 4·0).

(5) Same as No. 4, but *p*H 8·0.

(6) Pseudoglobulin (horse), defatted by Young's method, dissolved at *p*H 8·5, amylose added, globulin precipitated at *p*H 6·2; after filtering, the extent of combination was examined by determining the loss in concentration of amylose.

Table II.

Exp. no.	*p*H	Concentration of amylose %	Change in concentration of amylose %	Amylose bound by 100 g. of protein g.	Diminution between control and experiment mg.
1	4	0·11	7·7	0·8	0·5
2	7	0·11	8·1	0·9	0·5
3	5	0·1	13·1	1·2	2·4
4	4	0·11	3·7	0·45	0·9
5	8	0·11	3·1	0·4	0·8
6	6·2	0·15	0	0	0

It can be seen from this table, excluding Exp. 3, that pseudoglobulin and phosphoric acid-free amylose do not combine with each other. The values obtained are so small that they must be regarded as lying within the limits of experimental error. On the basis of these figures we can also conclude that the lipoid content of pseudoglobulin has only a small influence on the binding of amylose.

Binding of euglobulin with amylose.

The following experiments were made with euglobulin (ox):

(1) A suspension of undefatted euglobulin was prepared by dissolving at a high *p*H, then precipitated by the addition of HCl, and an amylose solution was added to the suspension. 25 ml. of 2 % suspension of euglobulin + 25 ml. amylose solution (*p*H 6·2)

(2) A solution of ox euglobulin, prepared as above, was taken; amylose was added and the protein then precipitated by the addition of HCl; 25 ml. of 2% solution of euglobulin + 25 ml. amylose solution (*p*H 6·2).

(3) An undefatted euglobulin solution was prepared as above; *N*/3 KCl was added and then amylose solution, after which the protein was precipitated by change of *p*H (*p*H 3·6).

(4) A solution of euglobulin, defatted by extraction with ether and methylene chloride and otherwise prepared as above, was taken, amylose was added and precipitation was effected at *p*H 5·0.

(5) A solution of ox euglobulin (defatted by Young's method) and prepared as above was taken; amylose was added and precipitation effected by change of *p*H (*p*H 6·2).

(6) Same as No. 5, but horse euglobulin used and *p*H 4·0.

It can be seen from Table III that it is the euglobulin constituent of globulin that causes strong binding with amylose, noticed in previous researches. Here, as with albumins, it is necessary to state that once the globulins are coagulated, they bind with amylose more weakly. It is also shown that fat has no significance in binding amylose. The fact that euglobulins extracted with ether and methylene chloride fail to bind with amylose requires further investigation.

Table III.

Exp. no.	*p*H	Initial concentration of amylose	Diminution in final concentration of amylose	Amylose bound with protein %
1	6·2	0·16	9·5	1·5
2	6·2	0·15	48·2	7·7
3	3·6	0·13	68·8	8·95
4	5·0	0·13	0	0
5	6·2	0·17	42·1	7·65
6	4·0	0·1	68·6	7·35

DISCUSSION.

Earlier research on the binding of proteins with polysaccharides showed that not all of the former bind the latter. The present research has made it possible to extend this observation, viz. it has been shown that under indentical conditions of binding the serum albumins bind amylose deprived of H_3PO_4 only in a very small degree, whilst of the globulins, only euglobulin binds, pseudoglobulin not doing so to any appreciable extent.

It can therefore be stated that the chemical composition of these two globulins is fundamentally different as the binding between polysaccharides does not depend (as was previously shown) on the colloidal properties of proteins (size and shape of molecules), but on their composition, i.e. on the composition of amino-acids, their distribution in the molecule and their ability to enter into the reaction.

It can also be confirmed that the bindings between proteins and amylose are not always of a heteropolar nature. De-lipoided proteins bind just as do those containing fat (cholesterol, lecithin, aliphatic acids). It hence follows that amylose is not bound by the lipoid groups attached to the protein.

It is of interest that coagulation of serum protein before the addition of amylose, even in the case of euglobulin with its strongly binding action, results in the binding being so weak that no certain conclusion as to its existence can be drawn. This is perhaps due to a mutual interaction of those groups which are responsible for combination with the amylose, or possibly also to their molecular grouping being re-arranged in such fashion that combination becomes impossible.

SUMMARY.

1. Phosphoric acid-free amylose is able to bind with proteins.

2. Euglobulin is the only serum globulin which binds with polysaccharides free of ionized groups; pseudoglobulin practically does not bind, whilst the albumins combine only in a very small degree.

3. Albumin coagulated by boiling or by change of pH does not bind amylose.

4. Fats do not affect combination between serum euglobulins and amylose, as those containing fat bind just as well as those deprived of it by Young's method.

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CCXV. A NOTE ON THE PRESENCE IN HUMAN PREGNANCY URINE OF AN ACID-HYDROLYSABLE COMBINED FORM OF PREGNANDIOL.

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PREGNANDIOL was isolated from human pregnancy urine by Marrian [1929] and shortly afterwards independently by Dingemanse *et al.* [1930] and by Butenandt [1930]. Its structure was determined by Butenandt *et al.* [1931]. Recently a revived interest has been shown in this compound as a result of the work by Butenandt & Schmidt [1934] who showed that by a simple series of procedures it can be converted into progesterone. In view of the obvious value of pregnandiol as a source of progesterone, it appeared to the authors to be advisable to make a study of the conditions necessary for its complete extraction from urine.

In previous work from this laboratory it has been shown [Cohen *et al.* 1935] that over 99 % of the oestrin in human pregnancy urine is in an acid-hydrolysable combined form. More recently it has been shown that the "combined" oestriol may be quantitatively extracted from the urine with butyl alcohol and that part, at least, of this is an oestriol monoglucuronide [Cohen & Marrian, 1936]. It has furthermore been shown [Schachter & Marrian, unpublished] that butyl alcohol will extract most of the "combined" oestrogens present in the urine of pregnant mares. It would also appear probable from the work of Adler [1934] that some of the androgenic material in human male urine is likewise present in a butyl alcohol-soluble, acid-hydrolysable "combined form". In view of these facts it seemed possible that pregnandiol might similarly be excreted in a butyl alcohol-soluble combined form.

EXPERIMENTAL.

The starting material in all these experiments was the butyl alcoholic extract of pregnancy urine which had been washed repeatedly with $N/3$ NaOH to remove the combined oestriol, washed with water and evaporated to dryness. Such a fraction would necessarily contain all the free pregnandiol originally present in the urine and probably any "combined" pregnandiol, provided that the latter was not sufficiently strongly acidic to be removed by the alkali washing.

The general procedure adopted was as follows.

The dry residue from the evaporation of the alkali-washed butyl alcohol was dissolved and suspended in a suitable volume of N NaOH. The ether-soluble neutral substances (including the free pregnandiol) were removed from this mixture by repeated ether extraction. The combined ethereal extracts, after washing with water, were evaporated to dryness and the residue dissolved in the smallest possible volume of hot acetone. The solution after 2–3 days at 0°, deposited crystals of crude pregnandiol. These were filtered off, washed with cold acetone and decolorized by boiling with charcoal in ethyl alcoholic solution. The material obtained by evaporation of the alcoholic filtrate was finally treated with warm light petroleum in which pregnandiol is nearly insoluble, in order to remove soluble impurities, and weighed. For final identification of this material by its melting point, it was recrystallized from the smallest possible volume of ethyl alcohol.

The original aqueous alkaline phase was then heated on a water-bath to remove all the dissolved ether, adjusted to the desired *p*H and hydrolysed by heating at 100° in a boiling water-bath or at 120° in an autoclave. The hydrolysis mixture was next made alkaline (where necessary) by the addition of NaOH, and repeatedly extracted with ether. Any pregnandiol set free by the hydrolytic treatment was isolated from this ethereal extract by the same procedure as described in the preceding paragraph.

1st experiment. From the butyl alcoholic extract of 100 litres of urine were obtained 46·7 mg. of crude free pregnandiol which after one crystallization from ethyl alcohol melted at 233–234°. After heating the aqueous alkaline phase at 100° for 4 hours only 1·2 mg. more of crude pregnandiol were obtained. The aqueous phase after hydrolysis for 1·5 hours at *p*H 2·0 at 100° yielded only 1·0 mg. more of crude pregnandiol. After a further hydrolysis at *p*H 1·5 at 100° for 2 hours 20 min., 108·2 mg. of crude pregnandiol were obtained, which after one crystallization from ethyl alcohol melted at 230–232°. The mixed M.P. with highly purified pregnandiol (M.P. 236·7–237°) was 230–234°.

2nd experiment. The butyl alcohol-soluble fraction from 360 litres of urine which had previously been extracted with ether was dissolved in NaOH, extracted with ether and then divided into nine equal portions. Each portion was then subjected to a different hydrolytic treatment. The results are shown in the following table.

Hydrolysis	mg. crude pregnandiol
*p*H 1·5 at room temperature for 8 days	Negligible
*p*H 1·5 at 100° for 15 min.	3·2
*p*H 1·5 at 100° for 30 min.	0·3
*p*H 1·5 at 100° for 60 min.	0·7
*p*H 1·5 at 100° for 120 min.	36·7
*p*H 1·5 at 100° for 240 min.	29·0
*p*H 1·5 at 120° for 30 min.	1·5
*p*H 1·5 at 120° for 60 min.	46·3
*p*H 1·5 at 120° for 120 min.	78·4

DISCUSSION.

The experiments described conclusively prove that a considerable fraction of the pregnandiol excreted in human pregnancy urine is in a combined, ether-insoluble form. Since this combined pregnandiol remains in the butyl alcohol after repeated extraction with aqueous *N*/3 NaOH, it is evident that it is a much less strongly acidic compound than is oestriolglucuronide. Since the alkaline washings of the butyl alcoholic extract of pregnancy urine have not yet been examined thoroughly for the presence of combined pregnandiol, the possibility cannot be excluded that other more strongly acidic compounds of pregnandiol are present in this fraction.

A rough determination of the conditions necessary for optimum hydrolysis of this combined pregnandiol has been made. It was not readily hydrolysed by hot *N* NaOH, at *p*H 1·5 in the cold or at *p*H 2·0 at 100° after more than 2 hours. Considerable hydrolysis took place at *p*H 1·5 at 100° after 2 hours, but since the yield after a 4-hour hydrolysis was somewhat lower, it would appear possible that further hydrolysis under these conditions was accompanied by destruction. Hydrolysis in the autoclave at *p*H 1·5 for 2 hours gave considerably higher yields than were obtained at 100°. It seems possible that the reason for this may be that the destruction of pregnandiol in hot acid solution is oxidative in character, as is the case with oestriol [Cohen & Marrian, 1935], and that in the more anaerobic conditions of the hydrolysis in the autoclave the destruction was decreased.

The attention of the authors was first drawn to the possibility of a destruction of pregnandiol by hot acid by the fact that the melting point of the crude pregnandiol obtained in the acid-hydrolysis experiments usually ranged between 205 and 220°, whereas that of the crude pregnandiol extracted before hydrolysis was usually between 220 and 225°. At first it was thought that the low melting point of the pregnandiol released by hydrolysis might be due to admixture with other substances having similar solubilities in acetone and light petroleum which had also been liberated. It was found, however, that the melting point of pure pregnandiol could be depressed to below 220° by prolonged boiling in aqueous acid solution. Such an explanation was therefore unnecessary.

It must be admitted that these results do not yet provide a wholly satisfactory solution of the practical problem of obtaining larger yields of pregnandiol from pregnancy urine. The conditions of hydrolysis which would be necessary for maximum yields of pregnandiol from whole urine are likely to be quite different from those suitable for the relatively urea-free and unbuffered extracts used in these experiments. Furthermore, owing to the destruction by acid, much smaller yields of the pure compound are obtained by ethyl alcohol crystallization of the crude pregnandiol released by hydrolysis than can be obtained from the same amount of crude pregnandiol directly extracted without hydrolysis. It is anticipated that it will be possible to devise methods of hydrolysis which will be suitable for whole urine and which will not cause extensive destruction of the liberated pregnandiol. Owing to the impossibility of continuing this work at the present time, it was considered to be advisable to publish these preliminary results.

SUMMARY.

The presence of an ether-insoluble "combined" form of pregnandiol in the alkali-washed butyl alcoholic extract of human pregnancy urine has been detected. This complex is stable to hot alkali but is hydrolysed by hot acid. The optimum conditions for its hydrolysis by acids have been approximately determined.

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Note added 15 August, 1936. Since the completion of this paper Venning & Browne (*Proc. Soc. Exp. Biol. N.Y.* (1936), **34**, 792) have reported the isolation from human pregnancy urine of an oestriol monoglucuronide.

CCXVI. PHYSICAL CHEMISTRY OF LIPOIDS.

V. BEHAVIOUR OF KEPHALIN FROM HUMAN BRAIN TOWARDS ACIDS AND BASES.

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Whilst the reactions between proteins and acids and bases have been extensively studied, no similar systematic investigations have been conducted on lipoids. Yet some phosphatides have features that seem to make them specially fit objects for such investigations. The chemical structures of lecithin and kephalin are nearly completely elucidated, and they form stable colloidal solutions in electrolyte-free water. Finally, kephalin, like proteins, is an ampholyte, the absence of the ampholytic feature from lecithin providing an interesting subject of comparison. In the experiments to be reported here the physico-chemical behaviour of kephalin towards HCl and NaOH was studied.

Some observations on lecithin described by other authors have been confirmed on material derived from the same brains as the kephalin.

The origin, preparation and chemical properties of the kephalin used in these experiments have been the same as reported in a recent paper [Spiegel-Adolf, 1935]. The kephalin was obtained from human brains, cleaned from blood and meninges, dried at 37° and subsequently pulverized. This powder was extracted with ether and then treated with absolute alcohol. This procedure was repeated three times. The preparation thus obtained contained 2·64 % of nitrogen and 5·6 % of phosphorus. It was entirely soluble in water, ether and chloroform, insoluble in absolute alcohol and acetone. The conductivity of a 1 % aqueous solution is $K_{25} = 3{\cdot}10 . 10^{-4}$ mhos, the $c\mathrm{H} = 1{\cdot}38 . 10^{-6}$. After further purification by means of dialysis, the conductivity dropped to $9{\cdot}3 . 10^{-5}$ mhos, and the cH rose to $4{\cdot}71 . 10^{-6}$. Further attempts at purification by electrodialysis, which is so successful in the case of proteins, have not proved to be equally useful in the case of kephalin (and lecithin) solutions. When a positively charged membrane was used on the side of the anode, the final cH of the fluid under electrodialysis did not rise above $1{\cdot}88 . 10^{-4}$, at which, according to the findings summarized in Tables I and II, kephalin solutions should still be stable. Yet during the electrodialysis the whole of the kephalin (and lecithin) was precipitated on the positively charged membrane. The deposited kephalin could be brought again into aqueous solution which became decidedly more acid than the original solution ($c\mathrm{H}\ 9{\cdot}16 . 10^{-4}$ against $1{\cdot}38 . 10^{-6}$). As a chemical change in the kephalin could not be excluded, the only purification method used was dialysis. All experiments were made on fresh samples of kephalin, although, unlike lecithin [Spiegel-Adolf, 1932; Fischgold & Chain, 1934], kephalin did not show signs of ageing.

In the first series of experiments various amounts of HCl were added to samples of kephalin. With increasing concentrations of HCl the solutions became more opaque until finally precipitation occurred. At 0·02 N HCl in 1 % kephalin the solution becomes heterogeneous, though it is still stable. But while, contrary to Thierfelder & Klenk [1930], watery solutions of kephalin cannot be precipitated by centrifuging (for 25 min. at a rate of 2000–3000 rotations per min.), at HCl concentrations from 0·01 *N* upwards centrifuging becomes effective.

In the different HCl-kephalin mixtures determinations of the conductivity, *c*H, and viscosity were made. The results are summarized in Tables I and II.

Table I.

Final concentration of kephalin = 1 %; *fa*H = activity coefficient of HCl according to Scatchard; *N* = normality of HCl; *K* = specific conductivity.

Final concentration of HCl *N*	*c*H	*fa*H	Bound HCl $n - \frac{c}{fa\text{H}}$	K^{30}_{cor}	$K - K_{\text{HCl}}$
0	$1{\cdot}38 . 10^{-6}$	—	—	$3{\cdot}16 . 10^{-4}$	—
0·0025	$6{\cdot}44 . 10^{-5}$	—	—	$5{\cdot}08 . 10^{-4}$	—
0·005	$2{\cdot}53 . 10^{-4}$	0·937	$4{\cdot}73 . 10^{-3}$	$7{\cdot}01 . 10^{-4}$	$5{\cdot}83 . 10^{-4}$
0·01	$2{\cdot}28 . 10^{-3}$	0·921	$7{\cdot}52 . 10^{-3}$	$1{\cdot}39 . 10^{-3}$	$3{\cdot}2 \ . 10^{-4}$
0·015	$5{\cdot}90 . 10^{-3}$	0·900	$8{\cdot}45 . 10^{-3}$	$2{\cdot}50 . 10^{-3}$	$-2{\cdot}61 . 10^{-4}$
0·02	$1{\cdot}01 . 10^{-2}$	0·887	$8{\cdot}60 . 10^{-3}$	$4{\cdot}50 . 10^{-3}$	$-2{\cdot}8 \ . 10^{-4}$

Table II.

Kephalin in all samples = 1 %; temperature = 30°; t_0 = flowing time of water = 857 sec. /5.

Final concentration of HCl *N*	t_1 = flowing time in sec. /5		After 25 min. of centrifuging
	At once	After 24 hours	
0	1062	1041	Homogeneous
0·0025	1045	1038	,,
0·0050	1037	1040	,,
0·0075	1036	1045	,,
0·01	Inconstant	Over 1220	Heterogeneous
0·015	,,	Over 3000	,,

An examination of the results of the potentiometric *c*H determinations shows that kephalin neutralizes a certain amount of acid, and that this amount increases with increasing HCl concentrations to a maximum value; 1 g. of kephalin combines with 8·6–8·8 . 10^{-3} ml. *N* HCl. According to Jukes [1934] 1 molecule of kephalin in non-aqueous solutions combines with 1 molecule of acid. As Levine & West [1916] found for brain kephalin a molecular weight of 823·7, the normality of a 1 % solution ought to be 0·0124. The lower value found in these experiments suggests that not all amino-groups are freely accessible to the acid. This may be explained by the formation of colloidal aggregates in aqueous solutions.

Lecithin does not contain a free amino-acid group, yet with this exception it is very similar in structure to kephalin, so that some experiments with HCl were made with this substance.

The lecithin used in these experiments has been made from the same brains as were used for the preparation of kephalin. After the latter had been precipitated, the alcohol-ether filtrate was concentrated and precipitated by acetone. This manipulation was repeated three times. The resulting preparation gave opaque colloidal solutions in water; it was completely soluble in ether, absolute alcohol and chloroform at 37° and insoluble in acetone. The preparation contained 2 % nitrogen and 10·5 % phosphorus. It seems therefore that the preparation contains some nitrogen-free phosphatides such as have been described by Chibnall & Channon [1927] in plants. 2 % colloidal solutions of lecithin were prepared according to the method of Keeser [1924]. Conductivity and *c*H were measured in the solution, in the various lecithin and HCl mixtures and in the corresponding HCl solutions. The differences between calculated and measured values were considered as a measure of bound HCl. All measurements were duplicated on egg-lecithin solutions (Merck, egg-lecithin extra pure).

Table III.

Lecithin concentration in all samples = 1 %; I refers to human brain lecithin, II refers to egg lecithin.

Final concentration of HCl N		Conductivity			cH		
		Measured	Calculated	Difference	Measured	Calculated	Difference
0	I	8·81 . 10^{-5}	—	—	2·71 . 10^{-7}	—	—
	II	1·93 . 10^{-4}	—	—	1·88 . 10^{-4}	—	—
0·0011	II	3·31 . 10^{-4}	8·05 . 10^{-4}	4·74 . 10^{-4}	9·92 . 10^{-4}	1·31 . 10^{-3}	3·16 . 10^{-4}
0·003	II	—	—	—	2·15 . 10^{-3}	2·66 . 10^{-3}	5·1 . 10^{-4}
0·005	I	1·81 . 10^{-3}	2·39 . 10^{-3}	5·8 . 10^{-4}	3·53 . 10^{-3}	3·97 . 10^{-3}	5·7 . 10^{-4}
	II	7·63 . 10^{-4}	2·51 . 10^{-3}	1·75 . 10^{-3}	3·67 . 10^{-3}	4·16 . 10^{-3}	4·9 . 10^{-4}
0·0075	I	2·83 . 10^{-3}	3·53 . 10^{-3}	7·0 . 10^{-4}	5·90 . 10^{-3}	6·40 . 10^{-3}	5·0 . 10^{-4}
	II	2·44 . 10^{-3}	3·71 . 10^{-3}	1·27 . 10^{-3}	5·90 . 10^{-3}	6·33 . 10^{-3}	4·3 . 10^{-4}
0·01	I	3·72 . 10^{-3}	4·80 . 10^{-3}	1·08 . 10^{-3}	7·79 . 10^{-3}	8·43 . 10^{-3}	6·4 . 10^{-4}
	II	3·90 . 10^{-3}	4·86 . 10^{-3}	9·6 . 10^{-4}	8·27 . 10^{-3}	8·55 . 10^{-3}	2·8 . 10^{-4}
0·015	I	5·56 . 10^{-3}	6·88 . 10^{-3}	1·32 . 10^{-3}	1·16 . 10^{-2}	1·25 . 10^{-2}	9·0 . 10^{-4}
	II	6·17 . 10^{-3}	7·12 . 10^{-3}	9·5 . 10^{-4}	—	—	—

The results of Table III show that both samples of lecithin bind HCl, but that according to their chemical difference (as indicated by their different cH) the binding capacity reaches its maximum value at a different HCl concentration. The fact that lecithin in aqueous solutions binds HCl is rather unexpected, since according to Jukes [1934] lecithin is an internally neutralized compound and has no free amino-group. But systematic investigations of the salt-binding capacity of lecithin solutions [Spiegel-Adolf, 1936] have given evidence of the sorptive power of lecithin. Furthermore, former experiments on globulins [Spiegel-Adolf, 1930] have shown that acid binding can be supplemented by acid sorption.

The behaviour of kephalin and HCl is very similar to the behaviour observed with proteins and acid, except that computation, based on the cH and conductivity data, shows that the ionization of the kephalin-HCl complex appears to decrease gradually.

These results seem to suggest the existence of a compound of kephalin and HCl in solutions. However, Thierfelder & Klenk [1930] reviewing the results of the literature on HCl precipitates of kephalin came to a different conclusion. In order to elucidate this question, special experiments were undertaken.

1. A solution containing 0·25 % kephalin and 0·025 N HCl shows partial precipitation. A chloride determination in the filtrate, after destruction of kephalin with HNO_3, shows that it contains only 90 % of the original amount of chlorine. This suggests that about 0·0025 N HCl has been precipitated with a part of the kephalin.

2. The conductivity was measured in a 0·25 % kephalin solution, in a 0·025 N HCl solution, and in a solution containing 0·025 N HCl and 0·25 % kephalin. The sum of the conductivities of pure kephalin and pure HCl was larger than the conductivity observed in the solution containing both substances. This difference in conductivities was 10 times larger than the conductivity of the pure kephalin solution. Calculated in terms of HCl-normality, the apparent loss in conductivity corresponded to an amount of 0·0025 N.

These results seem to suggest that HCl combines with kephalin not only in solutions but becomes part of the precipitate when precipitation occurs. By repeated centrifuging and replacement of the watery acid layer by pure water, most of the acid can be eliminated. This is due probably to hydrolysis of the lipoid-acid compound. The acid kephalin flocculate, when redissolved in water, settled at an acid concentration which primarily did not interfere with stability in centrifuging, but neutralization with alkali restored the original behaviour. These findings about the formation of kephalin-HCl compounds are perhaps not entirely devoid of biological interest, thus e.g. Peters & Man [1934] claim that part of the chloride of the serum is combined with lipoid.

The results of the viscosity determinations made with an Ostwald viscosimeter at a constant temperature of 30° show with increasing concentration of HCl a steady drop in viscosity, which only begins to rise again at a stage preceding precipitation. The initial drop becomes less distinct if the mixtures stand for 24 hours. These observations on the changes of kephalin viscosities upon addition of acids are very similar to those reported by Handovsky & Wagner [1911] on lecithin under comparable conditions. But the behaviour of kephalin is in this respect markedly different from the behaviour observed in proteins, for with the latter increasing HCl concentrations produce at first a rise in viscosity and ionization.

Analogous determinations were made on mixtures of kephalin and NaOH. The results are summarized in Tables IV and V.

Table IV.

Final kephalin concentration – 1 %; *fa*OH = activity coefficient of NaOH; N = normality of the NaOH.

Final concentration of NaOH N	K^{30}_{cor}	cOH	*fa*OH	$N - \frac{c\text{OH}}{fa\text{OH}}$
0	$3{\cdot}12.10^{-}$	$3{\cdot}88.10^{-7}$	–	–
0·0025	$4{\cdot}24.10^{-}$	$2{\cdot}92.10^{-7}$	—	—
0·005	$5{\cdot}84.10^{-}$	$7{\cdot}56.10^{-7}$	0·928	0·004999
0·01	$7{\cdot}49.10^{-}$	$1{\cdot}81.10^{-6}$	0·901	0·0099
0·015	$10{\cdot}21.10^{-}$	$1{\cdot}42.10^{-5}$	0·882	0·0149
0·02	$13{\cdot}00.10^{-}$	$7{\cdot}50.10^{-5}$	0·860	0·0199
0·05	$57{\cdot}00.10^{-}$	$1{\cdot}17.10^{-2}$	0·819	0·0356

Table V.

The final concentration of kephalin is 1 %; temperature = 30°; t_0 = flowing time of water – 857 sec. 5.

Final concentration of NaOH N	0	0·005	0·01	0·015	0·02
At once	1010	1270	1369	1290	1218
After 1 hour	–	1059	1027	1003	969
After 2 hours	—	1018	984	979	952
After 24 hours	1010	963	944	937	922

Since the reaction of NaOH with kephalin may lead to some chemical changes of the kephalin, attempts were made to ascertain whether the highest NaOH concentrations used produce a splitting of kephalin. As a breakdown of the kephalin molecule by alkali ought to liberate fatty acids, conductivity methods could be used in order to trace an increase in conducting material.

Samples containing 1 % kephalin and 0·02 N or 0·05 N NaOH were exactly neutralized after 24 hours. The conductivities of these solutions were nearly identical with the sum of the conductivities of controls containing either pure kephalin or pure alkali. A slight decrease in the conductivities of the mixtures was explained by the inactivating effect of kephalin on the salt. A splitting of kephalin in the alkali concentrations used in Tables IV and V can therefore be disregarded. The optical changes in kephalin on addition of alkali proved to be reversible on neutralization.

An attempt was made to determine the binding capacity of kephalin for NaOH. According to the figures in Table IV the NaOH-binding capacity exceeds by far the combining power of kephalin for HCl. Apparently in excess of alkali a maximum value could also be reached.

Viscosity measurements of kephalin at varying NaOH concentrations reveal a great similarity in behaviour to proteins, especially globulins [Spiegel-Adolf, 1930]. Here too, with increasing amounts of NaOH, the viscosity increases and drops again on further addition of alkali. The maximum viscosity is reached at a much lower concentration of alkali than the maximum binding capacity. Yet all these changes occur in the first hours after mixing kephalin and alkali. After several hours of standing, an increase of alkali has uniformly produced a corresponding decrease of viscosity.

The fact that kephalin, though combining with acid like a protein, shows no increase in viscosity like lecithin may be of some interest to the biologist. The non-swelling of nervous substance in acids [Bauer, 1911; Spiegel, 1921] has been attributed by Hooker & Fischer [1912] to reactions of lecithin prevailing over the reactions of proteins. This explanation gains new support from the results on kephalin reported here. Thus according to Singer [1926] brain contains more kephalin than lecithin. Recent studies of Spiegel & Spiegel-Adolf [1936] have shown that in dead and in living brains the polarization indices—a convenient measure of the swelling and the permeability of tissues—show a marked decrease on addition of alkali, whilst they are unchanged or only minimally lowered in the presence of acids.

SUMMARY.

1. Purified kephalin from human brains was studied in regard to its reactions with HCl and NaOH.

2. Measurements of the conductivity and of the cH show that kephalin combines with HCl. In the presence of an excess of HCl 1 g. kephalin neutralizes $8{\cdot}6$–$8{\cdot}8 . 10^{-3}$ ml. N HCl. Further increase of HCl precipitates a compound of kephalin and HCl.

3. The viscosity of kephalin is decreased by additions of HCl and begins only to rise again at a stage preceding flocculation. At this stage only can kephalin be precipitated by centrifuging.

4. Kephalin neutralizes NaOH, the binding capacity increasing with the concentration of alkali.

5. In fresh mixtures of kephalin and NaOH of increasing concentrations, the viscosity passes through a maximum. After 24 hours, the samples show only a steady decline in viscosity with increasing NaOH concentrations.

6. Some biological aspects of these findings are discussed.

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CCXVII. THE RÔLE OF ENZYMES IN ACTIVATED SLUDGE AND SEWAGE OXIDATIONS.

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IN a previous paper [Wooldridge & Standfast, 1933] it has been shown that the oxygen absorption of sewage from aqueous solution saturated with air generally depends upon the presence of bacteria, although these bacteria need not necessarily be alive provided that certain of their oxidation enzymes are still active. Experiments with activated sludge, using the Thunberg-methylene blue technique [Wooldridge, 1933], indicated that sewage sludges possessed active oxidation-reduction enzymes, which appeared to be of importance in the various processes of purification of sewage. In 1932 [Wooldridge & Standfast, 1932] a short note summarizing the results of these experiments was published and it was tentatively concluded "that the most important factor in sewage purification is a series of catalysed oxidation-reduction reactions determined by bacterial enzymes present in either living or dead bacterial cells or liberated by them into the fluid of the reaction system". In the papers of this series, but particularly in the present and subsequent ones, evidence in support of this view is given. This evidence has been obtained by determining the absorption of oxygen by sewage or sludge or both by direct measurement in a Barcroft respirometer under different conditions, the basic conditions simulating, on a small scale, those applying to the activated sludge process of sewage purification.

The effect of sterilization. When sewage is shaken with air in a Barcroft respirometer a rapid absorption of oxygen is observed, the extent of this absorption depending largely upon the so-called "strength" of the sewage [Wooldridge & Standfast, 1936, 1]. If the sewage is previously sterilized either by heating it for 30 min. at 120° or by passage through a Seitz bacterial filter no oxygen will be absorbed provided that precautions are taken to keep the system sterile throughout the experiment. Similar results are obtained with activated sludge; thus the rapid oxidation of this material is entirely inhibited by previously autoclaving the sludge. Further, a sterile mixture of sludge and sewage exhibits no oxygen uptake whether the mixture itself is autoclaved or whether the constituents are sterilized separately and subsequently mixed.

This loss of ability of sewage systems to absorb oxygen when they are rendered sterile can be demonstrated equally well by treatment with disinfectants. For this purpose such oxidizing disinfectants as permanganate, iodine, hydrogen peroxide *etc.* are to be avoided and chemically inert reagents such as ether, toluene, chloroform *etc.* are used. In the experiments recorded in this paper, the bulk of the disinfectant, after its period of action on the sewage, was generally removed, first by separation of the aqueous and non-aqueous layers and then by removal of the remaining disinfectant from the treated sewage by gentle aeration at low pressure. Tests for bacterial sterility were carried out on all the treated sewages and in these early experiments (*cf.* Tables VIII and IX) it was invariably

Table I. *The inability of sterile sewage or sterile sludge to absorb oxygen.*

The sewage (C.S.) used in these experiments was filtered through filter paper and then allowed to stand overnight alone or in contact with the disinfectants given below. The autoclaved (C.S. 120°) and the Seitz-filtered (C.S. sf.) sewage were prepared from the untreated sewage by autoclaving at 120° for 30 min. or by passage through a Seitz filter respectively. The respirometers were shaken at 24°. In each right-hand cup were placed 3 ml. of the sewage mixture and in the left-hand cup 3 ml. of water that had been treated similarly to the sewage. Volatile reagents were removed as far as possible by evacuation. As usual carbon dioxide was absorbed by potash. The figures give the oxygen absorption in μl. at N.T.P. in 24 hours; the method of sterilization, if any, is given at the head of the columns.

C.S. untreated	C.S. 120°	C.S. sf.	10% ether	10% chloroform	1% toluene
240	2	0·5	4	4·5	1·5

10% phenol	2% formaldehyde	Untreated sludge	Sludge 120°	C.S. 120° + Sl. 120
4·5	3	610	2	3

found that when the sewage was sterile no oxygen absorption (within experimental error) took place (Table I). It would appear that little, if any, purely chemical oxidation took place with the sewages examined, for destruction of all oxidizable substances by every method of sterilization adopted is unlikely.

Effect of addition of non-sterile sewage upon oxygen absorption by sterile sewage. That the oxidizable material of a sewage has not been destroyed or removed to any great extent by the process of sterilization is readily shown by adding a small amount of non-sterile sewage to an autoclaved or a Seitz-filtered sewage, when a steady uptake of oxygen subsequently develops. The addition of the "seeding" of non-sterile sewage appears to initiate the process of oxidation, probably as the result of the bacteria added in the seeding. Sterilization possibly induces some change in the constitution of the original sewage, for the rates of absorption of oxygen by seeded sewages, sterilized by different means, sometimes differ significantly (Table II).

Table II. *Reactivation of sterile sewage by the addition of non-sterile sewage.*

The right-hand cups of the respirometers contained 1 ml. of the sterile sewage mentioned below. All cups contained 1 ml. of the sterile buffer and except for the sterile controls 0·1 ml. of a non-sterile crude sewage was placed in the side arms and tipped into the main bulk of the cups after preliminary shaking for 1 hour. The contents of each cup were made up to 3 ml. with sterile tap-water. Carbon dioxide was absorbed in all cups by potash. The figures represent the oxygen uptake in μl. at N.T.P. The temperature of the experiments was 22°.

Sewage	Sewage seeding	2	18	24	42	48	66	72	90	138 hr.
C.S. sf.	0	0	0	0	0	0	0	0	0	0
C.S. sf.	+	17	21	29	59	67	80	84	91	101
C.S. 120°	0	0	0	0	0	0	0	0	0	0
C.S. 120°	+	9·2	13	19	48	54	70	72	76	86

Oxidation of sterile sewage in the presence of sludge. It is to be expected that the rate of oxidation of sterile sewage upon the addition of a small inoculum of crude sewage would be slow, as the amount of bacteria or enzyme thus added is small. Sterile sewage, however, may be oxidized rapidly by the addition of activated sludge. If equal amounts of washed sludge are placed in the cups of a Barcroft respirometer and a quantity of sterile sewage is added to the sludge in one cup then a greater absorption of oxygen takes place in this cup than in the other. This difference, which is readily followed manometrically, is, in fact, a

Table III. *Oxidation of sterile sewage in presence of sludge.*

In these experiments all cups except those of the controls contained 1 ml. of sludge, 1 ml. of phosphate buffer *p*H 7·4 and 0·5 ml. of water. Each left-hand cup had 0·5 ml. of water and each right-hand cup 0·5 ml. of the sterile sewage, the C.S. sf. and C.S. 120° being placed in the side-arm and subsequently tipped into the main cup after preliminary shaking for 20 min. The sterile control respirometers had 1 ml. of water in their cups in place of the sludge. C.S. sf., C.S. 120° and Sl. 120° = Seitz filtered sewage, autoclaved sewage and autoclaved sludge respectively. Carbon dioxide was absorbed by potash. The figures represent the oxygen absorbed in µl. at N.T.P. by the sewage in the presence of sludge.

Sewage added (right-hand cup)	Presence of sludge (in both cups)	1	2	3	4	5 hr.
0·5 ml. non-sterile C.S.	·	50	90	114	122	123
0·5 ml. C.S. sf.	+	49	83	105	113	119
0·5 ml. C.S. 120°	+	53	67	75	77	79
0·5 ml. C.S. sf.	0	0	0	0	0	0
0·5 ml. C.S. 120°	0	0	0	0	0	0
0·5 ml. sludge 120	+	19	36	54	72	92
0·5 ml. sludge 120	0	0	0	0	0	0
0·5 ml. sludge 120 + 0·5 C.S. sf.	0	0	0	0	0	0

measure of the rate of oxidation of the "sterile" sewage in the presence of the sludge. The results given in Table III show this oxidizing activity of sludge towards sterile sewage. Autoclaved sludge does not absorb oxygen but, like sterile sewage, it still contains constituents which can be readily oxidized by enzymically active sludge. A mixture of autoclaved sludge with a Seitz-filtered sewage shows no oxygen absorption (Table III). From the experiments described above it appears that the greater proportion, if not the whole, of the oxygen-absorbing power of sewage and sludge depends upon the presence of living material or of agents, e.g. enzymes present in the sewage or sludge, that are equally easily destroyed.

Organisms as a source of enzymes in sewage oxidations. Activated sludge must be considered as a living system comprising many forms of life, the chief of which are normally bacteria and protozoa. As the oxidation of sewage probably depends upon the presence of living organisms or their enzymes, the ability of a number of bacterial suspensions (some prepared from organisms isolated from sewage, others from laboratory stocks) to oxidize sterile sewage has been examined (Table IV). Most bacteria, when present in large numbers, in pure or mixed populations effect oxidation of sterile sewage to some extent. Some bacteria, e.g. laboratory strains of *Staph. aureus* and *Strep. faecalis*, brought about very little oxidation of the sewage. The results of Table IV are not strictly comparable, as neither the sewages nor the density of the bacterial suspensions were standardized, although the bacterial suspensions usually contained approximately 5×10^9 cells per ml. The most active organisms were *Bact. alkaligenes*, *Proteus vulgaris*, *Pseudomonas pyocyanea* and *Ps. fluorescens*; *Bact. coli* appeared to be less active. These bacterial suspensions were similarly able to bring about the oxidation of autoclaved sludge. The question of the importance of protozoa in sewage oxidations is frequently discussed and opinions are divided as to whether they are useful or deleterious in processes of sewage purification. Certain protozoa, absolutely free from bacteria, have been examined to see whether they are able to effect oxidation of sewage. For this purpose Miss Robertson of the Lister Institute kindly supplied two strains of bacteria-free protozoa, *viz.*

Table IV. *Oxidation of sterile sewage or sludge by bacteria or protozoa.*

The sewages used in the experiments below were not identical throughout and therefore the rate of oxidation in the presence of any one organism cannot be compared strictly with that obtained by other organisms; large differences are probably significant as the suspensions were of approximately the same strength as determined by turbidity standards ($\simeq 5 \times 10^6$ standard of *Bact. coli*). The organisms used consisted of pure cultures either isolated from sewage or obtained from laboratory cultures, except when otherwise stated. The bacteria were grown on nutrient agar for 24 hours at 22° or 37°, were washed off with sterile quarter-strength Ringer solution, washed twice and resuspended in this Ringer solution. The protozoa were grown in special bacteria-free media and were kept free from these organisms. These suspensions were used much thicker than those of bacteria. Sludge 120°, C.S. 120°, and C.S. sf. signify respectively autoclaved sludge, autoclaved sewage and sewage after passage through a Seitz bacterial filter. All cups of the Barcroft respirometers contained 0·5 ml. of sterile phosphate buffer, *p*H 7·4, and 1 ml. of the organism used except in the control experiments 24, 25 and 26 (no organism) and experiments 21 and 22 (only 0·5 ml. so that 1 ml. of mixed organism could be used in experiment 23). 1·5 ml. of sterile sewage or autoclaved sludge suspension were added to the right-hand cups and the total volumes were made up in both cups to 3 ml. with sterile water. CO_2 was absorbed by KOH. The protozoal cups were examined for bacterial sterility at the end of the experiments and none showed evidence of bacterial contamination. The figures represent the oxygen absorption in μl. at N.T.P.

Organism; present in both cups	Sewage preparation in right cup	1	2	3	4	5 hr.
Bacteria from sewage.						
1. Mixed bacteria grown direct from sewage	C.S. 120	32	57	73	84	93
2. Organism No. 1	C.S. sf.	23	35	45	49	55
3. Organism No. 2	C.S. sf.	32	53	69	76	80
4. Organism No. 9	C.S. sf.	21	34	44	52	59
5. Organism No. 14	C.S. sf.	49	87	108	112	114
6. Organism No. 16	C.S. sf.	24	33	39	43	49
Bacteria from other sources.						
7. *Pseudomonas pyocyanea*	Sludge 120°	155	267	333	417	—
8. *Ps. pyocyanea*	C.S. 120°	83	116	145	169	—
9. *Ps. fluorescens*	Sludge 120	35	80	119	—	178
10. *Ps. fluorescens*	C.S. 120°	23	57	105	—	189
11. *Ps. fluorescens*	C.S. sf.	15	45	87	-	170
12. *Chr. prodigiosum*	C.S. 120	21	42	58	68	—
13. *Bact. coli*	C.S. 120°	16	28	39	49	—
14. *Bact. aerogenes*	C.S. sf.	28	52	80	103	127
15. *Proteus vulgaris*	C.S. sf.	54	96	137	—	215
16. *Staph. aureus*	C.S. sf.	1·5	2·1	3	3·6	4
17. *Bact. alkaligenes*	C.S. sf.	60	112	161	—	257
18. *Strep. faecalis*	C.S. sf.	6·5	13	19	20	20
Protozoa (bacteria free).						
19. *Polytoma uvella*	C.S. sf.	35	49	58	69	—
20. *Euglena gracilis*	C.S. sf.	11·5	24	38	51	—
Bacteria + Protozoa.						
21. *Ps. fluorescens*	C.S. sf.	56·6	95	123	-	204
22. *Polytoma*	C.S. sf.	71·4	91	104	—	122
23. *Ps. fluorescens* + *Polytoma*	C.S. sf.	119	165	209	—	257
Controls (typical).						
24. —	Sludge 120°	0	0	0	0	0
25. —	C.S. 120°	0	0	0	0	0
26. —	C.S. sf.	0	0	0	0	0

Polytoma uvella and *Euglena gracilis*, which could be cultivated in the absence of bacteria. Suspensions of washed protozoa in quarter strength Ringer solution were prepared and their ability to promote oxidation of sterile sewage was examined in the Barcroft apparatus. Although the rate of oxidation of sterile sewage by bacteria-free protozoa appears to be comparatively poor, it nevertheless is definite but less than that of most of the bacteria examined. Table IV contains the results obtained with the most active preparations used; the turbidity of these suspensions was considerably greater than that of the bacterial suspensions examined. (Protozoal suspensions usually had a turbidity equivalent to that of suspensions of *Bact. coli* containing $20–50 \times 10^9$ organisms per ml.) The ability of *Polytoma* to cause oxidation of sterile sewage, although feeble, can be shown by the anaerobic methylene blue technique [*v*. Wooldridge, 1933]. Thus a suspension of *Polytoma* that took 46 min. to reduce 1 ml. of a 1/5000 solution of methylene blue at *p*H 7·4 was able to effect the same reduction in 25 min. in the presence of 2 ml. of a Seitz-filtered sewage: the methylene blue was not reduced by the sterile sewage alone.

Table V. *A comparison of the rates at which various organisms cause oxidation of sterile sewage.*

In these experiments the Barcroft respirometer had equal quantities of organism in the cups; sterile sewage was added to the right cup only. The volume of the liquid was made up to 3 ml. with 0·5 ml. sterile buffer, *p*H 7·4, and sterile tap-water. The thickest suspensions of both the sludge and the organisms were in Ringer solution and were of such a strength that on drying on a boiling water-bath and in a hot air-oven at 98°, the dry weight obtained on cooling in a desiccator was 0·02 g. per ml. suspension. The figures represent the uptake of oxygen in μl. at N.T.P. by the sewage. CO_2 was absorbed by KOH.

Organism suspension	Dry wt. per ml. mg.	1	2	3	4	6	9	22 hr.
Sludge	20	80	116	141	156	—	—	—
	2	30	60	95	115	—	—	—
	0·2	9	20	29	38	—	—	—
Ps. fluorescens	20	82	93	100	106	—	—	—
	2	26	61	78	82	—	—	—
	0·2	10	20	35	41	—	—	—
	0·02	7	10	12	14	18	25	172
Bact. coli	20	20	29	33	37	—	—	—
	2	11	19	26	33	—	—	—
	0·2	6	10	17	22	—	—	—
	0·02	5·5	9	11	11·5	12·5	14	78
Chr. prodigiosum	20	29	39	45	49	—	—	—
	2	14	27	39	45	—	—	—
	0·2	2	5	9	13	—	—	—
Staphylococcus	20	3	4	5	6	—	—	—
	2	2	3	3	4	—	—	—
	0·2	0	0	0	0	—	—	—
Polytoma uvella	20	14	19	20	21	—	—	—
	2	3	4	5	7	—	—	—

A direct comparison of the oxidative powers of a few of these organisms towards the same sterile sewage was made by using suspensions containing equal amounts of the various cells as measured by the dry weight of the suspensions (Table V). The activity of sludge was most nearly approached by that of *Pseudomonas fluorescens*: *Bact. coli* and *Chromobacterium prodigiosum* were less active and a laboratory strain of a staphylococcus showed very little oxidizing activity. *Polytoma* showed an oxidizing power somewhat greater than that of the staphylococcus, but less than that of *Bact. coli*. The weakest suspensions of *Bact. coli* and *Pseudomonas fluorescens* show little difference in the first 4 hours but after that, probably because of a greater ability of the latter organism to proliferate in the sewage, the difference between their oxidizing powers becomes greater. Previous results, obtained with the test for biochemical oxygen demand in 5 days, have tended to exaggerate somewhat these differences between different organisms, for no oxygen was absorbed from solution when *Bact. coli* was added to the sterile sewage to an extent of 0·02 mg. per ml. whilst the corresponding addition of *Ps. fluorescens* caused the oxygen to be almost completely removed (> 16 precipitate 10^5). This difference was no doubt due to the fact that *Ps. fluorescens* grew well during the experimental period of 5 days, thereby producing a large amount of active enzymes, whilst *Bact. coli* died off without causing absorption of a measurable quantity of oxygen [*v*. Wooldridge & Standfast, 1933].

Butterfield *et al.* [1931], working with a dilute peptone-sugar medium in place of a sewage, brought forward evidence to show that protozoa, e.g. *Colpidium*, by keeping down the numbers of bacteria stimulate the bacteria to further growth and thus increase the rate of absorption of oxygen so that the value obtained for the mixture of bacteria and protozoa is greater than that of the sum of the two acting separately. The effect of mixing pure suspensions of *Ps. fluorescens* and *Polytoma uvella* has been examined with the Barcroft apparatus but no increased oxidation of the sewage as a result of the presence of the protozoa with the bacteria at least during the few hours of this experiment (Table IV, exps. 21, 22 and 23) was found. It is nevertheless possible that the mixed population would prove to be more active when it had settled down into equilibrium.

Addition of urethane to oxidizing sewage and sludge. The previous experiments appear to indicate that sewage or sludge is not oxidized by air in the absence of living organisms, especially bacteria. Many bacteria [Quastel & Whetham, 1925; Quastel & Wooldridge, 1925] possess enzymes, such as dehydrogenases, that will effect the oxidation of a variety of substances under suitable aerobic or even anaerobic conditions. Wooldridge [1933] has shown that sewage sludges possess some of these enzymes and that sewage usually contains constituents in solution that are able to act as substrates for these enzymes. The results given in Tables VI and VII testify that, as with oxidations by bacterial dehydrogenations, sewage oxidations as observed in the Barcroft apparatus are entirely inhibited by the presence of 5 % urethane.

Addition of cyanide to oxidizing sewage and sludge. Wooldridge [1933] showed that activated sludge possesses indophenol oxidase besides various dehydrogenases and suggested that both types of enzyme were important in sewage oxidations. If this is so then, as the activity of indophenol oxidase is completely inhibited by $M/1000$ KCN, this concentration of cyanide should arrest the absorption of oxygen by sewage. Experiment [Table VII] indicates that the uptake of oxygen by crude sewage or by sludge is greatly inhibited by this and greater concentrations of cyanide, particularly for the first few hours. Gradually, however, the inhibiting effect appears to wear off, probably as a result of changes which the cyanide itself undergoes. The inhibiting effect of cyanide can be shown quite readily by placing solid KCN in the side-arm of the Barcroft cup, when apparently sufficient HCN diffuses over into the fluid to inhibit the oxidation. It is perhaps surprising that the effect is so soon demonstrable, for it is apparent as soon as the manometers are closed. In this way a continuous supply

Table VI. *The effects of cyanide and of urethane upon the oxidation of crude sewage.*

The right cup of the Barcroft respirometer contained 2 ml. crude sewage, 0·5 ml. phosphate buffer *p*H 7·4 and 0·5 ml. of water or of the inhibitor, the strength of which was such that its final concentration was as stated below. The cyanide solutions were previously neutralized with HCl to *p*H 7·4. The contents of the left cup were the same except that the sewage was replaced by water. The solid KCN was placed in the side-arm of the cups and was not in direct contact with the fluid at all. KOH papers were present to absorb CO_2. The figures represent the absorption of oxygen in μl. at N.T.P. Viable counts were done on the contents of the cup of the crude sewage and the solid KCN experiments at the end of the experiment.

	3 hr.	5 hr.	7 hr.	11 hr.	21 hr.	27 hr.	Viable count bacteria
Crude sewage alone	130	235	291	366	451	545	15×10^6
C.S. + solid KCN in side-arm	36	36	37	38	41	42	$0{\cdot}9 \times 10^6$
C.S. + $M/200$ KCN	10	12	14	18	125	310	—
C.S. + $M/500$ KCN	15	15	21	26	175	321	—
C.S. + $M/1000$ KCN	15	21	25	56	345	410	—
C.S. + $M/10{,}000$ KCN	60	165	274	350	440	520	—
C.S. + 5% Urethane	—	—	3	—	5	5	—

Table VII. *The effects of cyanide and of urethane on the oxidation of activated sludge.*

1 ml. of a suspension of washed sludge was placed in each right-hand cup of the respirometers. 0·5 ml. of buffer, *p*H 7·4, was put into each cup and the contents were made up to 7 ml. with the addition of water or inhibitor solution so that the strength of the latter was as stated in the table. The solid KCN was placed in the side-arms out of contact with the fluid. The figures give the oxygen uptake in μl. at N.T.P. CO_2 was absorbed as usual by KOH. Final bacterial counts were made on the suspensions of sludge in the control and solid KCN experiments, and both gave figures of approximately 1 million per ml. Experimental temperature, 21°.

	1 hr.	2 hr.	3 hr.	5 hr.	7 hr.	21 hr.	Viable count bacteria
Sludge alone	33	52	70	103	136	338	3×10^6
Sludge + solid KCN in side-arm	6	13	18	26	33	70	3×10^6
Sludge + $M/10$ KCN	7	11	12	17	20	—	—
Sludge + $M/100$ KCN	11	18	26	38	45	—	—
Sludge + $M/1000$ KCN	15	29	38	64	86	—	—
Sludge + $M/10{,}000$ KCN	22	32	52	79	103	—	—
Sludge + 5% urethane	—	—	—	—	1·3	3	—

of cyanide is available and the inhibiting effect continues for long periods. In order to discover whether this inhibition is due to sterilization of the medium, bacterial counts were made at the end of the experiments on the crude sewage, and on the sewage and sludge mixture with solid KCN in the side-arms. It was found that although the number of viable bacteria was reduced in the case of sewage and cyanide to about a sixteenth of those present when the cyanide was absent yet there were still approximately a million viable organisms per ml. whilst with sludge the fall in numbers seemed inappreciable. The viable count on the sludge mixture must be accepted with reserve as any change in the number of viable organisms actually enmeshed in, or adsorbed on, the sludge would not be determined. In both experiments, however, the number of viable organisms present at the end was sufficient to make it appear unlikely that the effect of the cyanide is solely or even largely a bactericidal one.

Bacterial viability and oxygen uptake of sewage and sludge. It has previously been reported [Wooldridge & Standfast, 1933] that sterile sewage, incubated under the conditions of the test for biochemical oxygen demand, will not absorb dissolved oxygen but that it will do so if heavily inoculated with a suspension of *Ps. fluorescens* previously treated with formalin so that many of the bacterial oxidizing enzymes remain active although the organism has lost the power of proliferation. Attempts to extend this work to a demonstration of oxygen absorption in the Barcroft apparatus have been only partially successful. The results given in Table VIII illustrate that formaldehyde-sterilized bacteria are capable of inducing the oxidation of sterile sewage; the oxygen uptake is small, *viz.* about 20 μl. per ml. of sewage in 5 hours, but significantly different from the controls. Sterilization of the sewage by other disinfectants always resulted in destruction of the ability to absorb oxygen. Irradiation with ultraviolet light sterilizes and ultimately destroys the power of sewage to oxidize in the presence of air, but if the irradiation be carefully controlled the sewage may be rendered sterile while still able to absorb oxygen. It is clear from the results given in Table IX, where each experiment was made in quintuplet, that the sterile

Table VIII. *The oxidation of sterile sewage by formaldehyde-treated bacterial suspensions.*

The suspensions used had an opacity ≡10 × 10^7 cells of *Bact. coli* per ml. The suspension was treated overnight at room temperature with the concentration of formaldehyde given below. All right-hand cups contained 1 ml. of autoclaved sewage and 1 ml. of phosphate buffer (sterile) was placed in all cups. 1 ml. of the treated bacterial suspension was added to each cup of the respirometer as given below. Other experimental details as in previous tables. Each experiment was made in quadruplicate and the mean results are given below. The replicates differed little one from another. The sterility of the mixtures in the cups was tested at the beginning and end of the experiment.

	HCHO concn.	μl. O_2 absorbed at N.T.P. in hours					Viable bacteria	
Organism	%	1	2	3	4	5	Beginning	End
Ps. fluorescens	0·36	9·3	14	17·4	20·4	22·2	0	0
,,	0·15	10·1	14·3	18·6	23·1	26·3	0	0
,,	0·05	17·1	26·1	33·3	34·2	34·8	+	+
None	0	6	7	7·5	8	8	0	0
Mixed sewage	1·1	9	13	14·7	16·1	16·9	0	0
,,	0·8	11·4	16	18	20·1	21·2	0	0
,,	0·55	11·4	15·8	18·2	20·5	21·3	0	+
None	0	3	3·8	4·8	5	5	0	0

Table IX. *Oxygen absorption by sewage sterilized by irradiation with ultraviolet light.*

The sewage was sterilized by irradiation with ultraviolet light for 75 min. at a distance of 4 in. The irradiation was given in three periods of 25 min. with 10 min. intervals. The temperature of the sewage, which was gently stirred during irradiation, did not rise above 42 . The right-hand cups of the respirometers contained 3 ml. of the sewage mentioned below and the left-hand cups contained 3 ml. of tap-water. CO_2 was absorbed by KOH. All cups were sterilized at the beginning of the experiment, and 0·5 ml. of the contents of the right-hand cups was added to 5 ml. of broth at the end of the experiment in order to test for viable organisms. The results given are the mean of the readings of 5 replicate respirometers.

Sewage	ml. oxygen absorbed in 3 hours	Growth on inoculation into broth
Crude	37·6 (S.E. = 1·25)	+ + +
Irradiated with ultraviolet light	27·5 (S.E. = 2·08)	0
Autoclaved	0·5 (S.E. = 3·48)	0

irradiated sewage absorbed oxygen almost as rapidly as did the crude sewage. Cook & Stephenson [1928] showed that bacteria killed by irradiation with ultraviolet light may retain the activity of oxidizing enzymes when they are able to absorb oxygen in the presence of suitable substrates, and so the uptake of oxygen by irradiated sewage is probably due to the fact that although the bacteria have been killed all their oxidizing enzymes have not been destroyed.

Identical suspensions of bacteria were irradiated with ultraviolet light for variable periods of time in order to obtain suspensions with the same total number of cells but with different numbers of viable organisms. The abilities of these treated suspensions to induce oxidation of sterile sewage were determined by the Barcroft apparatus, and the results compared with the viable counts obtained by examining the contents of the cups at the beginning and at the end of the experiments, the counts being made by the agar roll tube method [Wilson, 1922]. The results are given in Table X. The number of viable organisms did not increase, but, in fact, decreased somewhat during the course of the experiment, and hence bacterial proliferation is an unlikely explanation of the results obtained. The viable organisms in the initial counts varied from 5635×10^6 down to 20×10^6 per ml. yet at the end of 2 hours the oxygen absorption was the same; even the initial rate of oxidation shows no direct correspondence with the viable count as is seen from the fact that the oxygen uptake in 30 min. with 5635×10^6 organisms was only 6 times that with 20×10^6 organisms although the ratio of the viable organisms present was 282 to 1. This experiment clearly indicates that the oxidation of sterile sewage can be induced by bacterial cells that are not regarded as being alive by ordinary standards of proliferation.

Table X. *Bacterial viability and sewage oxidations.*

The suspension of washed *Ps. fluorescens* used had a turbidity equivalent to that of a *Bact. coli* suspension of 30×10^9 cells per ml. 5 ml. volumes of this suspension were exposed to irradiation by ultra-violet light in quartz bulbs 4 in. away from the lamp for the times mentioned below. The temperature of the bulbs was kept low by submitting them continuously to a current of cold air. 0·5 ml. of the bacterial suspension, when used, was placed in each cup of the manometer, and 2 ml. of the Seitz-filtered sewage were placed in all right-hand cups. 0·5 ml. sterile buffer, *p*H 7·4, was in all cups. CO_2 was absorbed by KOH. All experiments were made in quadruplicate and checked by the method recommended by Corbet and Wooldridge [1936].

Exposure to U.V.L.	V.C. in cup		Oxygen uptake in μl. after			
min.	Original	Final	30 min.	60 min.	90 min.	120 min.
0	5635×10^6	3760×10^6	41	63	73	86
5	1250×10^6	340×10^6	44	70	82	87
10	125×10^6	47×10^6	29	64	72	77
15	20×10^6	7×10^6	7	31	59	80
0	0 (Sterile sewage only)	0	3·5	4	4·6	5

DISCUSSION.

No attempt has been made, in this series of papers, to study the clarification stage of the activated sludge process of sewage disposal but much work has been carried out upon the second stage of oxidation. In effect this process is a complex of oxidations of the numerous constituents found in sewages, and practically the whole of these oxidations would seem to depend upon biological factors. Sterile sewage and sterile sludge generally undergo no oxidation but when micro-organisms such as certain bacteria or protozoa are added the oxidation will take place.

Different organisms are not necessarily equally effective and no doubt the activity of any particular organism will depend upon the nature of the sewage, i.e. whether it contains substances which can act as substrates for the particular enzymes available, and also upon the condition of the oxidative enzymes of the organism. Wooldridge *et al.* [1936] have shown that the activity of these bacterial enzymes may vary considerably with varying conditions. The protozoa examined exhibit less activity than that of the bacteria, but even if the protozoa, under the conditions of the activated sludge process, were more active than appears from these experiments, their activity, in comparison with that of the very much more numerous bacteria usually present, must in practice be of secondary importance. The results have not demonstrated increased activity resulting from mixing protozoa and bacteria together but it may be that these experiments, which were only allowed to proceed for 5 hours, were too short to show the effects reported by Butterfield *et al.* [1931].

It has been shown previously [Wooldridge, 1933; Wooldridge & Standfast, 1932 and 1933] that sewage and sludge possess such enzymes of oxidation as indophenol oxidase and dehydrogenases, and it was suggested that these enzymes are mainly bacterial in origin. Previously Keilin [1929] indicated an important co-ordinating mechanism by which many cells possessing both these systems can utilize molecular oxygen in their cellular oxidations. The co-ordinating mechanism is an "oxygen carrier" and is a substance, e.g. cytochrome, which can exist in both an oxidized and a reduced form, the two being readily interconvertible. Molecular oxygen is activated by the indophenol oxidase and the activated oxygen reacts with the reduced form of the carrier. The resulting oxidized form of the carrier diffuses away from the oxidase and may reach a dehydrogenase, where it will react with the activated substrate of the dehydrogenase, reverting again to the reduced form of the carrier. In this way the molecular oxygen is used indirectly to oxidize the substrate of the dehydrogenase. The view that this or a similar mechanism operates in sewage oxidations is supported by the experiments which show that either cyanide or urethane inhibit these oxidations, for cyanide inhibits the oxidase but not the dehydrogenases while the urethane inhibits dehydrogenases but not indophenol oxidase.

The view that enzymes, as distinct from the whole living organism, are responsible for many of these oxidations is supported by these inhibition experiments, and also by the experiments with formaldehyde-treated organisms, with crude sewage carefully sterilized by irradiation with ultraviolet light, and with bacterial suspensions irradiated with ultraviolet light which show that the oxidation depends more upon the presence of active enzymes than upon the number of viable organisms present. Although these experiments clearly indicate that bacterial enzymes, not associated with living organisms, can be effective in causing oxidation of sewage and sludge the enzymes of living and especially of actively proliferating organisms will also be active and, mass for mass, are likely to be more active than those present in dead cells. With the activated sludge process however the amount of enzymically active material associated with the preponderating numbers of dead cells making up the bulk of the sludge is probably very great and hence the oxidizing activity of this material is likely to be of considerable importance in the oxidations effected by activated sludge.

SUMMARY AND CONCLUSIONS.

1. The work described in this paper supports the view previously put forward that the most important factor in biological oxidations of sewage is a series of catalysed oxidation-reduction reactions determined by bacterial enzymes present in either living or dead bacterial cells or liberated by them into the fluid of the reacting system.

2. Crude sewage, sterilized by filtering through a Seitz bacterial filter, by heat or by various "disinfectants", does not usually absorb oxygen when shaken in a Barcroft microrespirometer (cf. 5 and 6 below). Autoclaved sludge does not absorb oxygen, even on vigorous aeration.

3. Addition of a seeding of crude sewage, a suspension of washed sludge, a suspension of washed bacterial cells (of several species) or a suspension of bacteria-free protozoal cells induces the oxidation of sterile sewage or sterile sludge. Some species of bacteria effect the oxidation of sterile sewage rapidly but with others the rate is comparatively slow. Two species of protozoa have been examined and these are less active than most bacteria. A mixed suspension of *Ps. fluorescens* and *Polytoma uvella* caused no more rapid oxidation of the sterile sewage than the sum of the rates obtained when the bacterium and the protozoon were allowed to act separately.

4. Urethane (5 %) inhibits oxidation of sewage and sludge. Cyanide ($M/200$ to $M/10{,}000$ KCN, neutralized, or the vapour from solid KCN) inhibits the uptake of oxygen by crude sewage and by sludge but the inhibition is not complete and usually lessens with time, presumably due to the alteration of the cyanide.

5. Bacteria carefully treated with formaldehyde, although unable to proliferate on the usual nutrient media, are nevertheless capable of inducing a definite though slow oxidation of sterile sewage.

6. Sewage sterilized by irradiation with ultraviolet light will still absorb oxygen on shaking with air provided the irradiation is not too severe.

7. Several originally identical suspensions of *Ps. fluorescens*, irradiated with ultraviolet light for different periods of time so that the number of viable cells differed considerably from suspension to suspension, oxidized sterile sewage at rates largely independent of the proportion of viable cells present.

8. Oxidation of the constituents of sewage and of sludge depends generally upon the presence of certain oxidative enzymes (dehydrogenases and oxidases) of micro-organisms. These enzymes may be effective whether the organisms are alive or dead, provided that the method of killing has not destroyed the enzymes. Although protozoa possess enzymes that can oxidize the constituents of sewage, it is concluded that the greater proportion of the oxidation is brought about by bacterial enzymes, the bacteria being both far more numerous and generally much more active.

9. Oxidations of sewage depend mainly on the presence of bacterial enzymes, which themselves may be associated with either living or dead cells. No direct comparison has been made between the enzymic activities of living and dead cells but it is probable that the activity of the living proliferating cell for certain oxidations is greater than that of the dead cell. The dead cell is important in oxidations by activated sludge as the sludge is likely to contain a large proportion of dead bacterial cells many of which are probably enzymically active.

This investigation was carried out as part of the programme of research of the Water Pollution Board of the Department of Scientific and Industrial Research. Thanks are due to the Borough Engineer of Finchley for the regular supply of the samples of sewage used in this work.

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CCXVIII. A COLORIMETRIC METHOD FOR THE DETERMINATION OF CHOLINE AND ACETYLCHOLINE IN SMALL AMOUNTS.

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While the presence of small amounts of choline and acetylcholine can easily be detected by biological methods, their quantitative micro-chemical estimation presents considerable difficulty. The only method hitherto described for the micro-chemical estimation of choline appears to be that of Roman [1930], a modification of the macro-method of Stanek [1906] depending on the precipitation of choline as choline periodide. By this method quantities of choline from 0·001 to 5 mg. can be estimated, but its application is limited since creatinine, adenine and other nitrogenous bases are also precipitated. Furthermore, the method is complicated by the fact that the physical conditions of the experiment require very careful control. In the present paper a simple colorimetric method for the estimation of choline and acetylcholine is described.

The principle of the method is the same as that used by Kapfhammer and Bischoff [1930] for the isolation of choline and acetylcholine from tissues. It depends on the fact that choline and acetylcholine form reineckates which are insoluble in ice-cold water and in absolute alcohol but are easily soluble in acetone giving a bright red solution. In the method of Kapfhammer and Bischoff acetone was used on account of its selective solvent action on the reineckate, for the further purification of the precipitate after washing with ice-cold water and absolute alcohol. The acetone was then evaporated and after several extractions with ether the insoluble residue was dried and weighed.

In the present method the reineckate is dissolved in acetone, and by comparing the colour of this acetone solution with that of a standard acetone solution of choline or acetylcholine reineckate, the amount of choline or acetylcholine combined as reineckate can be determined. The depth of the red colour depends on the amount of the reineckate radicle present. The colours are the same in equimolecular acetone solutions of choline and acetylcholine reineckates, since 1 mol. of each base combines with 1 mol. of reineckate.

By this method quantities as small as 0·2 mg. of choline chloride and 0·32 mg. of acetylcholine bromide can be determined.

Method.

The solutions used were:

1. Choline chloride solutions containing 0·2–2 mg. choline chloride per ml.
2. Acetylcholine bromide solutions containing 0·3–3 mg. acetylcholine bromide per ml.

1 ml. of the solution is taken and to it 1 ml. of a freshly prepared saturated solution of ammonium reineckate (B.D.H.) is added. A precipitate of choline reineckate or acetylcholine reineckate separates out. These two reineckates differ in appearance and solubility. The choline reineckate has a glistening appearance;

its precipitation is complete in less than 10 min. and it can be quantitatively precipitated at temperatures up to 60°. The acetylcholine reineckate separates more slowly as a dull pink powder and for complete precipitation requires a much lower temperature (about 5°). To ensure that the precipitation is complete the container should be surrounded by ice for 20 min. A means of avoiding the greater difficulty in estimating the acetylcholine is to estimate the choline set free by alkaline hydrolysis. This hydrolysis can be effected in a few min. at room temperature by addition of NaOH to 1% concentration, the solution being neutralized before precipitation. After precipitation is complete the solution is filtered by suction through a small asbestos-packed crucible and the precipitate on the asbestos is washed once with 2 ml. of ice-cold water and twice with 2 ml. of absolute alcohol, or until the alcohol is no longer pink. The receiving flask is emptied and a test-tube is placed inside the flask to receive the acetone solution. Before applying suction 1 ml. of acetone is poured on the precipitate, which rapidly dissolves. The acetone solution is sucked into the receiving tube and successive small amounts of acetone are added until all the precipitate is dissolved and washed through. The tube is removed from the filtering flask and the contents are transferred to a graduated tube or small measuring cylinder which can be read to 0·05 ml. The receiving tube is rinsed with acetone and the washings added to the graduated tube. The volume of the acetone solution is carefully noted and the colour is compared with that of a standard solution containing 0·78 mg. of choline reineckate per ml of acetone or with a methyl red solution of the same colour, the preparation of which as a standard is described later. The standard corresponds to 0·26 mg. choline chloride or to 0·42 mg. acetylcholine bromide per ml. When the total amount of choline is small (0·2–0·3 mg.) the total volume of the acetone should not be more than 1–2 ml. in order to have a colour comparable with that of the standard. The comparison of the colour in such a small volume can be carried out in a micro-colorimeter. When on the other hand the total amount of choline is large (2–3 mg.), dilutions up to 10 ml. are required to obtain a colour comparable with the standard. For colorimetric comparisons daylight was found to be more suitable than artificial light.

Calculation. With the standard set at 20 mm. and the unknown giving a reading of x, then $\frac{20}{x} \times 0{\cdot}26$ (for choline) × volume of the unknown solution = mg. of choline chloride in amount of solution taken.

Preparation of standard solution.

A sample of choline reineckate was prepared according to the method of Kapfhammer and Bischoff, the only difference being the use of ammonium reineckate instead of reinecke acid for the precipitation. The total N of the dried choline reineckate determined by Kjeldahl's method was 22·8% (calc. 23·2%).

78 mg. of this salt were dissolved in 100 ml. of acetone. This solution was unsuitable as a permanent standard owing to evaporation of the acetone and fading of the colour. An artificial standard was therefore prepared by dissolving 0·0134 g. of methyl red in 5 ml. of $N/5$ NaOH and diluting to 500 ml. with distilled water; for use 4 ml. of this stock solution were transferred to a flask, 10 ml. of $N/100$ H_2SO_4 were added and the volume made up to 100 ml. A pink solution was obtained of the same colour as the 0·078% acetone solution of choline reineckate, corresponding to 0·26 mg. choline chloride per ml. or 0·42 mg. acetylcholine bromide per ml. The dilute standard remained unchanged for some days and the stock solution indefinitely. Several different samples of methyl

red were used in the preparation of standard solutions and the colours obtained in each case were the same.

Examples of the results obtained by this method for the choline estimation in choline chloride solutions are given in Table I, col. 2. The values are compared with those obtained by Roman's method [1930], col. 3, and with those calculated as choline chloride from the total N, col. 1.

Table I.

mg. choline chloride per ml.

1 Calculated from total N	2 Colorimetric estimation	3 Roman's method
2·35	2·35	—
1·56	1·52	—
0·90	0·92	0·93
0·88	0·87	—
0·76	0·70	—
0·72	0·71	0·71
0·70	0·67	—
0·62	0·62	—
0·54	0·54	0·59
0·46	0·48	—
0·35	0·38	—
0·31	0·32	—

Table II gives the results obtained by the estimation of acetylcholine precipitated at 5° as acetylcholine reineckate from acetylcholine bromide solutions.

Table II.

mg. acetylcholine bromide per ml.

Calculated from total N	Colorimetric estimation
1·90	1·87
1·26	1·28
0·99	0·96
0·49	0·43
0·40	0·38

Effect of temperature.

The comparative solubilities of choline and acetylcholine reineckates at different temperatures (5–60°) are shown by the following experiments.

1 ml. of acetylcholine bromide solution was precipitated with ammonium reineckate at room temperature (16°) before and after alkaline hydrolysis in 1 % NaOH and the acetylcholine chloride values of the two solutions were compared with that obtained when 1 ml. of acetylcholine bromide solution was precipitated at 5°. The results are given in Table III.

Table III. *Effect of temperature on the estimation of acetylcholine bromide.*

Acetylcholine bromide. mg. per ml.

Temperature ° C.	(Before alkaline hydrolysis)	(After alkaline hydrolysis)
5	1·28	—
16	0·97	1·26
19	—	1·26
60	Nil	1·17

Using a choline chloride solution containing 1·35 mg. per ml. the choline reineckate precipitation was not affected by increasing the temperature up to 60°.

These values, typical of many, show that with a solution of acetylcholine reineckate low values are obtained if the temperature is allowed to rise, whilst the yield of choline reineckate is not diminished at any temperature up to 60°. By hydrolysing the acetylcholine to choline before precipitation temperature effects can be minimized and accurate determinations carried out at room temperature. It was noticed that acetylcholine when in the presence of choline chloride can also be quantitatively precipitated at room temperature.

Effect of dilution on precipitation of choline.

A series of estimations was made in solutions containing from 0·3 to 0·03 mg. choline chloride per ml. To each solution 1 ml. of saturated ammonium reineckate was added and the precipitate in each case was dissolved in 2 ml. of acetone. The full value was obtained at all these dilutions. The temperature was kept low (5°) during the procedure since the extreme range of the method was being tested. At greater dilutions than the above the choline values fell. It thus appears that a quantitative precipitation of free choline can be obtained in solutions containing as little as 0·03 mg. choline chloride per ml. Since the final precipitate must contain about 0·2–0·3 mg. choline chloride in order to be comparable with the standard. 7–10 ml. of such a solution must be taken for the precipitation. 1 ml. of saturated ammonium reineckate solution is sufficient for the precipitation of quantities of choline up to 3 mg. choline chloride.

Effect of dilution on precipitation of acetylcholine.

An acetylcholine bromide solution corresponding to the final dilution of choline chloride at which a quantitative precipitation was found possible was treated in the same way but at a lower temperature (1–2°). The precipitation was carried out before and after alkaline hydrolysis. The results are given in Table IV.

Table IV.

10 ml. solution containing 0·48 mg. acetylcholine bromide	mg. acetylcholine bromide found
Before hydrolysis	0·28
After hydrolysis	0·50

At this dilution the acetylcholine cannot be quantitatively precipitated even at low temperatures. This demonstrates the advantage of hydrolysis of the acetylcholine to choline before precipitation.

The results obtained by this method show that quantities of choline chloride from 0·2 mg. upwards can be determined with an error of ±3%. Precipitation may be carried out at room temperature unless the solution is very dilute (containing 0·03–0·05 mg. per ml.), in which case a lower temperature (5°) is necessary for quantitative precipitation. For the estimation of acetylcholine it is advisable to hydrolyse to choline before precipitation since the precipitation of choline reineckate is less influenced by temperature than that of acetylcholine reineckate. In the same way after suitable hydrolysis the amount of choline in other choline compounds can be determined. As an example of this the results obtained on acid hydrolysis of a lecithin emulsion are given in Table V. The emulsion examined had total N 0·046 mg. per ml. It was hydrolysed in 7·8%

HCl at 110° and after different intervals the amount of free choline was determined. The results are expressed in terms of choline-N. These show the rate of liberation of choline and the percentage of the total N which can be accounted for by choline.

Table V. *Lecithin emulsion hydrolysed in 7·8 % HCl at 110°.*

Time in hours	mg. choline-N per ml.
0	—
4	0·012
9	0·022
21	0·036

The emulsion used was made from a commercial egg lecithin preparation (Griffin & Tatlock). The maximum value of free choline was obtained after 21 hours' hydrolysis, at which time the choline-N amounted to 78 % of the total N in agreement with Maclean and Maclean [1927].

The results given so far have dealt with the estimation of choline and its compounds in pure solution. To see how far the choline estimation may be interfered with by the presence of other substances such as occur in animal tissues and fluids, known amounts of choline chloride were added to extracts of different organs (rabbit's liver and kidney), to a tryptic digest and to urine, and the choline value was determined before and after addition. The results are given in Table VI. The kidney and liver extracts were prepared by extracting 1 vol. of liver or kidney with 3 vol. of 5 % trichloroacetic acid, and subsequently filtering and neutralizing. The tryptic digest was acidified, boiled, cooled and filtered before precipitation.

Table VI.

mg. choline chloride per ml.

	1 Originally present	2 Amount added	3 Total found	4 Recovered
Kidney extract	0·13	0·69	0·85	0·72
Liver extract	0·18	0·77	0·94	0·76
Tryptic digest	Nil	1·50	1·56	1·56
Urine	Nil	1·50	1·56	1·56

From these results it is seen that added choline can be recovered from crude extracts without appreciable loss. It follows that the method is applicable to the study of the liberation of choline from choline compounds in tissue extracts by chemical or enzymic hydrolysis. The original choline value of the reineckate precipitate from such an extract represents both choline and acetylcholine. Strack *et al.* [1935] have recently pointed out that the value obtained by Bischoff *et al.* [1932] for the acetylcholine content of skeletal muscle is probably a carnitine value, since this base also forms a reineckate with properties similar to those of acetylcholine reineckate. In order to find whether carnitine would also be estimated in the present method, a preparation of carnitine was made from a meat extract according to a method described by Strack *et al.* [1936]. The gold salt was prepared, the gold removed, and a reineckate precipitated which was found to be insoluble in water and alcohol, and soluble in acetone giving a pink colour. Carnitine therefore, if present, will also be estimated with choline in this method as in that of Bischoff and Kapfhammer. Since its amount in ordinary tissue extracts is small, no error will arise from this source in the study of the breakdown of choline compounds.

SUMMARY.

A micro-colorimetric method for the determination of small amounts of choline is described. Quantities of the order of 0·3 mg. choline in a concentration of 0·003 % can be estimated with an error of not more than 3 %.

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CCXIX. STUDIES ON THE PARTITION OF SERUM CALCIUM.

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IN a previous communication [Godden & Duckworth, 1935] we recorded certain data as to the partition of serum-Ca at or about the time of calving in the case of normal cows and of cows suffering from milk fever. Apart from these results no record appears to exist in the literature of systematic studies on the serum-Ca partition of individual animals throughout a reasonable period of their life span.

Since Benjamin & Hess [1933, 1] and Benjamin [1933] first presented their barium sulphate adsorption method of serum-Ca fractionation several writers have expressed views contrary to those of these two workers. We wish therefore to give the results which we have obtained as part of an investigation on the effect of certain food constituents on the growth and health of sheep, together with some physico-chemical studies of these data, in the hope that these new results may help to elucidate the problem.

EXPERIMENTAL.

Six half-bred wether lambs were divided into two groups. Nos. 25 and 26 constituted the control group and Nos. 21, 22, 23 and 24 the starch-fed group. The animals were kept indoors throughout the experiment in concrete pens bedded with sawdust. Both groups received the same basal ration consisting of turnips 3 lb., linseed cake meal 6 oz., chopped straw 8 oz., these amounts being just sufficient, when fed daily, to maintain the control group in practically steady body-weight. The starch-fed group received, in addition to this basal ration, a gradually increasing amount of maize starch, which was adjusted in amount to the appetite of the group. For the control group the daily intake of Ca was 1·84 g. and of P 1·92 g. For the starch-fed group, owing to a trace of P in the starch, the intake of P was slightly higher reaching a maximum of 2·04 g. Thus the respective Ca:P ratios were 1:1·04 and 1:1·11, a negligible difference. The vitamin D content of the diet was low, no cod-liver oil being added.

The experiment lasted approximately 6 months, the animals being weighed individually at fortnightly intervals. Blood samples were drawn at approximately three-weekly intervals. The blood was drawn from the jugular vein, employing partial stasis and the following determinations were made:

(1) Total serum-Ca. The method of Clark & Collip [1925].

(2) Serum-Ca partition. The method of Benjamin & Hess [1933, 1, 2]. The membranes were prepared by the method previously described by one of us [Duckworth, 1935].

(3) Serum inorganic P. The method of Bodansky [1932–33].

(4) Serum protein. Kjeldahl method.

Table I. *Serum analyses, including Ca partition. All results expressed in mg. per 100 ml.*

Sheep no.	Dates of sampling									
	14. xii. 34	23. i. 35	12. ii. 35	25. ii. 35	13. iii. 35	1. iv. 35	15. iv. 35	23. iv. 35	18. v. 35	4. vi. 35
					Total Ca.					
21	9·3	8·2	6·2	6·0	5·5	6·0	6·0	6·5	7·5	7·3
22	9·5	8·8	7·6	6·6	5·7	7·2	7·1	6·5	7·4	7·3
23	9·6	8·7	8·0	5·8	6·7	7·1	5·6	5·4	7·6	7·0
24	9·7	8·9	9·1	8·0	7·3	8·4	7·7	7·8	8·0	7·6
25*	8·7	9·9	8·8	9·2	9·4	10·6	10·1	10·1	10·4	10·6
26*	10·6	10·8	10·2	10·0	10·6	11·1	10·3	11·1	11·0	10·3
					Ca^{++}.					
21	1·4	0·9	0·6	0·5	0·6	0·6	0·6	0·8	1·1	0·7
22	1·4	1·2	0·9	1·0	0·6	0·8	1·1	0·7	1·2	1·1
23	1·7	1·1	1·1	0·5	0·8	0·6	0·5	0·5	1·1	0·8
24	1·5	1·2	1·0	1·0	0·7	1·0	0·8	1·1	1·5	1·1
25*	1·5	1·5	1·0	1·2	1·2	—	1·2	1·2	1·6	1·1
26*	2·1	2·1	1·6	1·8	1·8	2·1	1·3	2·1	2·4	1·3
					Ultrafiltrable Ca complex.					
21	3·4	4·2	3·0	3·0	2·5	1·7	2·5	3·0	3·3	3·6
22	3·9	4·5	3·2	2·8	2·2	2·4	3·0	2·2	3·2	2·7
23	3·5	4·1	3·6	3·0	2·6	2·6	2·7	2·6	3·5	3·4
24	3·4	4·4	4·4	4·1	3·3	3·2	3·5	3·1	3·2	3·3
25*	3·3	4·4	4·2	4·4	3·9	—	4·7	4·6	4·6	4·7
26*	3·9	5·0	4·5	4·8	4·5	4·8	5·1	4·5	4·2	4·9
					Non-ultrafiltrable Ca complex.					
21	1·6	1·2	1·3	1·3	1·5	2·2	1·9	1·5	1·5	0·5
22	1·4	1·0	1·1	1·5	1·9	2·5	1·2	2·3	1·1	0·9
23	1·9	1·5	1·0	1·3	2·1	2·5	1·5	1·7	1·3	0·2
24	2·4	1·4	1·3	1·3	2·2	2·4	2·0	2·4	1·4	1·4
25*	1·0	1·9	1·6	1·9	2·5	—	2·3	2·6	1·9	2·0
26*	2·0	1·0	1·6	1·7	2·5	2·1	2·0	2·0	1·8	2·1
					Protein-bound Ca.					
21	2·9	1·9	1·3	1·2	0·9	1·5	1·0	1·2	1·6	2·5
22	2·8	2·1	2·4	1·3	1·0	1·5	1·8	1·3	1·9	2·6
23	2·5	2·0	2·3	1·0	1·2	1·4	0·9	0·6	1·7	2·6
24	2·4	1·9	2·4	1·6	1·1	1·8	1·4	1·2	1·9	1·8
25*	2·9	2·1	2·0	1·7	1·8	—	1·9	1·7	2·3	2·8
26*	2·6	2·7	2·5	1·7	1·8	2·1	1·9	2·5	2·6	2·0
					Serum inorganic P.					
21	6·48	6·74	8·00	8·24	6·80	9·24	6·06	7·84	6·56	7·84
22	6·31	5·28	4·64	5·46	5·74	5·08	3·32	5·38	4·50	3·42
23	4·59	6·06	4·74	9·24	5·88	6·64	7·10	10·60	5·64	4·38
24	6·48	6·68	7·36	7·36	8·68	5·96	6·78	7·32	5·54	4·76
25*	5·81	7·04	7·16	7·62	7·36	6·16	4·66	8·56	7·22	7·70
26*	5·52	5·34	5·72	6·40	6·16	5·06	4·86	5·02	3·92	4·96
					Serum protein.					
21	—	—	5·09	4·94	5·67	5·69	5·85	5·64	—	—
22	—	—	5·85	5·48	5·54	5·86	5·87	6·06	—	—
23	—	—	5·85	5·50	5·40	5·78	5·96	5·38	—	—
24	—	—	5·38	5·09	5·37	5·99	5·56	6·06	—	—
25*	—	—	4·79	5·27	5·19	5·95	5·62	5·31	—	
26*	—	—	5·29	5·34	5·39	5·73	5·56	5·62	—	—
					*p*K Ca proteinate.					
21	—	—	2·09	2·10	2·06	2·41†	2·13	2·03	—	—
22	—	—	2·11	2·06	2·21	2·28	2·04	2·27	—	—
23	—	—	2·04	2·01	2·21	2·27	2·02	2·25	—	—
24	—	—	2·07	1·99	2·15	2·18	2·11	2·10	—	—
25*	—	—	2·13	2·05	2·18	—	2·07	2·11	—	—
26*	—	—	2·07	1·95	2·07	1·99	2·00	2·06	—	—

* Denotes control animals. † Figure probably erroneous and not included in calculations.

For the sake of brevity the following abbreviations for the four serum Ca fractions are used throughout this paper.

Ca^{++} = Ca ion.
U.Ca.C. = ultrafiltrable Ca complex.
N.U.Ca.C. = non-ultrafiltrable Ca complex.
P.B.Ca. = Protein-bound Ca.

The analytical results for all the samples are shown in Table I.

It is of considerable interest that the animals tolerated such reduced Ca^{++} levels with no apparent symptoms of heart disorder. Stewart & Percival [1928] have claimed that Ca in complex form is capable of furnishing Ca^{++} at the surface of the heart cell. Further reference to the data of this experiment will be made in the discussion.

Results.

It is not proposed, in this paper, to discuss the clinical observations or the practical significance of these data in relation to animal nutrition. It should be noted, however, that at the conclusion of the experiment, the "knock-kneed" condition, generally supposed to be rickets, had developed in sheep Nos. 21, 22 and 23, while No. 24 appeared to be normal. At the sampling on 4. vi. 35 this condition had become severe. It is remarkable that it was only when both complexes showed *simultaneous* low values that severe leg-weakness occurred. Of the starch-fed group only No. 24 appeared normal and in this case the N.U.Ca.C. was only slightly depressed.

Effect of depression of the Ca^{++} upon serum-Ca partition.
(In vivo *and* in vitro *experiments.*)

(*a*) In vivo *depression of the Ca^{++}.* In this experiment five successive intra-jugular injections of 100 ml. each of a warm 20% solution of sodium citrate ($Na_3C_6H_5O_7,2H_2O$) were given to a normal, mature Ayrshire cow. The injections were made subject to changes in the condition of the animal rather than on a regular time interval basis. The blood samples were drawn from the jugular vein on the opposite side to that in which the injections were made. All analyses were made on serum.

Analytical methods.

(1) Total serum Ca and Ca partition as in the sheep experiment.

(2) Serum Mg. On the supernatant fluid from the serum Ca estimation, by precipitation as $MgNH_4PO_4$ and colorimetric estimation by the method of Fiske & Subbarow [1926].

(3) Inorganic P. Method of Fiske and Subbarow.

Table II. *Injection of sodium citrate into the jugular vein of a normal cow.*

Values in mg. per 100 ml. of serum.

Sample no.	Time of sampling a.m.	Time of injection a.m.	Inorg. P	Mg	Total Ca	Ca^{++}*	U.Ca.C.	N.U.Ca.C.	P.B.Ca.
1	10·01	10·03	5·13	3·00	11·5	2·3	4·0	0·6	4·6
2	10·06	10·11	5·00	3·00	11·4	3·0	4·0	0·6	3·8
3	10·16	10·20	3·24	3·16	12·0	4·7	3·1	1·0	3·2
4	10·25	10·29	4·91	2·94	11·2	4·5	3·6	0·5	2·6
5	10·35	10·40	4·91	2·83	11·2	4·5	3·3	0·5	2·9
6	10·44		5·59	2·63	10·4	4·5	3·3	0·5	2·1

* Under Ca^{++} is also included the Ca bound as citrate.

Results.

Immediately following each injection there was a response followed by a recovery period. With each successive injection the magnitude of the response increased and the animal became unconscious after the fifth injection.

The final effect of repeated citrate injections was a slight depression of the total serum Ca. The apparent rise and final high values of Ca^{++} are fictitious. Ca citrate is not adsorbed by $BaSO_4$ and therefore, under the system of analysis, appears in the Ca^{++} fraction. The values for Ca^{++} really represent mixtures of Ca^{++} and Ca citrate. There appears to be little doubt that the concentration of Ca^{++} was gradually decreasing if the increase in severity of the clinical symptoms can be taken as indicative. Further, the gradual and almost regular fall in P.B.Ca., ending in a final value of less than half the pre-experimental level, shows clearly the mobilization of P.B.Ca. in an endeavour to remedy the Ca^{++} deficit caused by the binding action of the citrate ions.

The two complexes do not show corresponding mobilizations. In fact it might appear that mobilization from the N.U.Ca.C. did not occur. In this connection it should be noted, however, that the animal was showing, at the pre-injection sampling, a lower value for this fraction than we have ever found for a normal animal of any species. On the other hand, the U.Ca.C. did show evidence of some slight mobilization, there being a final drop of about 15%. From our observations we would say that, whilst some of the clinical symptoms of this animal were similar to those occurring in milk fever, the complete picture did not parallel what we have noted in such cases. We have previously pointed out [1935] that, in cases of true milk fever, there are *simultaneous* low levels for both adsorbable and non-adsorbable Ca. In this experiment there was a definite fall in the non-adsorbable Ca (i.e. Ca^{++} + P.B.Ca.) but the adsorbable Ca (i.e. U.Ca.C. + N.U.Ca.C.) showed relatively little change (maximum fall 18%) and the final values were markedly above those which we cited for cases of milk fever.

The other values do not call for particular comment.

(*b*) In vitro *depression of the* Ca^{++} *in serum.* 30 ml. aliquots of a bulk serum were added rapidly with shaking to increasing amounts of sodium citrate ($Na_3C_6H_5O_7,2H_2O$) dissolved in 1 ml. of water and left overnight. The Ca fractionation was performed the next morning. The results of one of a series of such experiments are shown in Table III.

Table III.

All results in mg. per 100 ml.

Sample no.	Na citrate in mg. per 100 ml. of serum	Total Ca	Ca^{++}	U.Ca.C.	N.U.Ca.C.	P.B.Ca.
1	0	10·8	2·4	4·3	0·7	3·4
2	47	10·8	3·4	4·0	0·8	2·6
3	93	10·8	4·2	4·0	0·6	2·0
4	143	10·8	5·2	3·6	0·6	1·4
5	190	10·8	5·9	3·2	0·6	1·1
6	240	10·8	6·6	2·9	0·5	0·8
7	287	10·8	7·3	2·9	0·2	0·4

The progressive increase in the Ca^{++} values does not indicate a real increase in Ca^{++} because, as before mentioned, the method employed includes Ca citrate with Ca^{++}; in presence of citrate ion true Ca^{++} would undoubtedly be diminished. The steady mobilization of P.B.Ca. is obvious and this fraction has practically disappeared with the highest citrate concentration. Of greatest interest is the slow mobilization of the Ca in complex form, both complexes gradually liberating Ca^{++} but not to such an extent as the P.B.Ca.

Stewart & Percival [1928] studied the effect of sodium citrate on the diffusibility of serum Ca in relation to the time of coagulation, but do not state clearly which hydrated salt they used. Our results confirm theirs in that we found, with increasing concentrations of citrate, that the serum Ca gradually becomes more and more diffusible. They further report that the time required for blood to clot, when gradually increasing amounts of citrate were added, decreased at first and then increased until no coagulation occurred. The failure to coagulate preceded somewhat the rendering of the Ca almost completely diffusible and this they interpreted as evidence of a non-diffusible Ca complex essential for the normal coagulation of blood and evidence further that the Ca^{++} had no effect on the process of blood clotting. We suggest that a more probable explanation is that the P.B.Ca., which acts as a circulatory reserve of readily available Ca^{++}, had been completely mobilized and was therefore incapable of liberating more ions to remedy the deficit caused by citration. It may be noted that these authors found that inhibition of coagulation occurred when the citrate concentration, calculated on the serum, was between 250 and 330 mg. per 100 ml., a result which, assuming that they used the dihydrate, coincides with our value, 287 mg., for almost complete mobilization of P.B.Ca.

DISCUSSION.

Theories as to the physico-chemical state of Ca in body fluids may be divided into two groups: (*a*) that all the Ca not combined with protein is ionic, (*b*) that the Ca not combined with protein is partly in non-ionic forms. Our results lead us to support the second alternative.

Maclean & Hastings [1934, 1, 2; 1935, 1, 2] and Maclean *et al.* [1934] have published comprehensive studies on Ca partition. They believe that the amplitude of the contraction of the ventricle of an isolated frog heart preparation is proportional to the Ca^{++} concentration of the perfusing fluid. They maintain that practically all the serum Ca not in combination with serum proteins is ionic. They then calculate the constant, K, from the mass law equation for the dissociation of Ca proteinate, assuming that the protein molecule behaves as though it were composed of a series of negatively charged divalent ions, i.e.

$$\frac{[Ca^{++}]\,[\text{Protein}^{=}]}{[\text{Ca proteinate}]}=K.$$

The concentrations are in mols.

We have tested the validity of this expression as applied to our own results. We assumed, as did Maclean & Hastings, that grams of protein can be converted into milliequivalents by the factor for serum proteins, 0·243, found by Van Slyke *et al.* [1928]. In converting milliequivalents into millimols we have accepted their hypothesis that serum proteins act as divalent anions towards Ca. We have combined the values for Ca^{++} and U.Ca.C. as if all the ultrafiltrable Ca were ionic, and combined the N.U.Ca.C. and P.B.Ca. as non-ultrafiltrable Ca.

Our expression then becomes

$$\frac{[\text{U.Ca.}]\,[\text{Protein}^{=}]}{[\text{N.U.Ca.}]}=K.$$

The $[\text{Protein}^{=}]$ was obtained by difference between [Total protein] and [N.U.Ca.]. The results, expressed as the negative logarithms (pK), are given in Table I.

Statistically considered we find that $pK=2\cdot10$ with an S.D. $= \pm 0\cdot08$. Our actual figures show a pK range of 1·95 to 2·28 and hence a K range of 0·01131 to 0·00533 Taking only the S.D. the corresponding ranges are pK 2·02–2·18 and K 0·00966–0·00664. Maclean & Hastings give a mean pK of $2\cdot22 \pm$ S.D. 0·07, neither figure being far removed from ours. These values would give a K range of 0·00708–0·00513, while their complete K range would be correspondingly greater. We feel that dissociation constants with such ranges as are shown in both these sets of figures are of doubtful value and that the presence of such variability is, in itself, evidence of other influencing factors, which are not considered in the expression. We do not deny, but rather affirm, that an equilibrium between ultrafiltrable Ca and at least a part of the non-ultrafiltrable Ca does exist but we would add that the phosphate and bicarbonate ions play a rôle in this equilibrium.

Maclean & Hastings have constructed a nomogram which gives the Ca^{++} concentration of serum from the serum proteins and total serum Ca. Andersch & Oberst [1936] found that, in the case of the new-born child the ultrafiltrable Ca values as determined were about 20% lower than the Ca^{++} values for the serum (as determined by the nomogram). The agreement was closer for the serum of non-pregnant, pregnant and parturient women. Further, Benjamin & Hess [1933, 2] have recorded figures in the case of hypercalcaemia of egg-laying in fowls from which the pK, when calculated, shows a value of 3·04 with a corresponding value for K of 0·00091.

The values found for the Ca^{++} concentration of serum by Brinkman and van Dam [1920] using the solubility product method, by Neuhausen and Marshall [1922] using the flowing Ca electrode, and by the $BaSO_4$ adsorption method are in general agreement. The value obtained is, in all cases of the order of 2 mg. per 100 ml. Further, our own studies on Ca partition in pathological cases, the results of which will be published elsewhere, show a correlation between the clinical symptoms and the Ca partition data, which we find impossible to regard as fortuitous. Consequently we conclude that the four fractions of Ca found by the $BaSO_4$ adsorption and ultrafiltration methods represent real entities.

SUMMARY.

1. The results of studies in the Ca partition of sheep on experimental rations are recorded along with some results of physico-chemical investigations of the blood of cattle following both *in vitro* and *in vivo* addition of sodium citrate.

2. The results are considered as demonstrating that the so-called dissociation constant of Maclean and co-workers for Ca proteinate in serum is too variable to be regarded as an aid to the study of Ca partition.

3. It is concluded that the four Ca fractions found in serum by the barium sulphate method of adsorption coupled with ultrafiltration are real entities.

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CCXX. THE CHEMICAL COMPOSITION OF TEETH.

IV. THE CALCIUM, MAGNESIUM AND PHOSPHORUS CONTENTS OF THE TEETH OF DIFFERENT ANIMALS. A BRIEF CONSIDERATION OF THE MECHANISM OF CALCIFICATION.

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BECAUSE of the significance usually attached to the determination of ash, Ca and Ca/P ratio in bones and teeth in relation to diet and disease, it is important to give due consideration to the interrelationship of the Ca and Mg contents and the ash values. Orent *et al.* [1934] have studied the question of Mg deficiency in rats and shown how the Mg content of the bones varies with age in control animals and in animals on diets deficient in Mg. These differences in Mg content caused related inverse changes in Ca content and resulting changes in ash content (1 g. Mg $\equiv$ 1·648 g. Ca).

Having determined the Ca/P ratio in numerous samples of human teeth, where the values fell between 1·92 (lowest dentine) and 2·153 (highest enamel), it was surprising to find values as low as 1·7 and 1·8 in rats' incisor teeth, although rats' and rabbits' bones gave the expected ratios of 2·13 to 2·15, confirming the generally accepted values. This led to the investigation of the amount of Mg present in dental tissues from various sources and to the correlation of the results with the Ca and P contents.

Many of the usual phosphate precipitation methods for Mg determination were tried but finally rejected in favour of the hydroxyquinoline method of Greenberg & Mackey [1932]. This method adapted to determinations in solutions of dental material proved very satisfactory with an error of about $\pm 0{\cdot}5\,\%$. Table 1 gives the results of Ca, Mg and P determinations on the ash of various dental tissues. The Ca/P ratios were calculated. The Mg values were converted into their equivalent amounts of Ca, which values were then added to the corresponding actual Ca contents to give "Total Ca". Using these total Ca values, "Corrected Ca/P" ratios were calculated.

It was decided to compare the values of the rodents' whole teeth with the dentine of horse, dog and human, rather than with the enamel. The differences in composition between dentine and enamel have been dealt with in a previous publication [Bowes & Murray, 1935]. Enamel has on an average 0·4 % Mg.

In the consideration of the results, certain points relating to the theories of calcification, which have been dealt with fully by Robison [1932], have been borne in mind. The generally accepted view is that calcification takes place in calcifying tissues from solutions rendered saturated or supersaturated when the product ($Ca^{++} \times PO_4^{\equiv}$) exceeds the solubility product. The material deposited, "the bone salt", is an insoluble phosphate of the apatite series $3Ca_3(PO_4)_2 . CaX_2$.

Table I.

Material	% in ash Ca	Mg	P	Ca/P	Mg expressed as Ca	"Total" Ca	Ca/P "Corrected"
Rabbit incisors	35·02	2·467	19·88	1·761	4·061	39·08	1·965
Rabbit molars	35·76	1·45	20·15	1·775	2·39	38·15	1·893
Hare incisors	35·33	2·304	20·16	1·753	3·797	39·13	1·941
Hare molars	36·41	1·788	19·25	1·891	2·947	39·36	2·045
Guinea-pig incisors	35·80	1·745	19·56	1·831	2·876	38·68	1·978
Guinea-pig molars	35·01	1·932	20·04	1·748	3·185	38·20	1·903
Rat 12 weeks incisors	36·20	2·243	19·68	1·840	3·697	39·90	2·027
Rat 15 weeks incisors	34·82	2·363	19·62	1·774	3·895	38·72	1·973
Rat 1 year ditto	38·85	1·741	20·01	1·917	2·869	41·72	2·085
Horse dentine	37·12	1·855	19·23	1·931	3·058	40·18	2·101
Dog dentine	37·61	1·545	19·27	1·952	2·547	40·16	2·095
Elephant dentine (tusk)	32·91	4·055	19·64	1·675	6·681	39·60	2·016
Human dentine* sound, permanent, newly erupted, premolars	39·83	1·175	19·04	2·091	1·936	41·77	2·193
Human dentine sound, permanent, old (Indian)	37·81	1·310	18·46	2·048	2·165	39·97	2·165
Human dentine sound, temporary	37·64	1·391	18·18	2·071	2·293	39·93	2·189
*Corresponding enamel	39·98	0·424	18·42	2·171	0·70	40·68	2·206

Robison [1932] considered that in all probability the other inorganic constituents of bones and teeth can be incorporated into this complex molecule, equivalent amounts of other bases replacing the Ca and various groups CO_3, (OH_2), Cl_2 and F_2 representing the X_2. The Ca/P ratio of such a compound is 2·153. Replacement of one Ca by Mg would give a ratio of 1·935, the same as that of tricalcium phosphate $Ca_3(PO_4)_2$. If Mg does replace Ca in the apatite of bones and teeth, then having calculated the amount of Ca equivalent to the Mg, the Ca/P ratio should approximate to that of apatite. Orent *et al.* [1934] have shown that Mg and Ca do replace one another in rats' bones.

The results in Table I show that the human dentine contained the least Mg and gave the highest Ca/P ratio both actual and corrected. The corrected value was close to that of apatite. Next in order came the horse and dog dentine with corrected ratios differing a little from that of apatite. The P values of human, dog and horse dentine differed very little from one another. Taken as a whole the results from these dentines agreed reasonably with the theory that the calcified substance is largely apatite. Human enamel corresponds very closely to apatite. The elephant tusk dentine (ivory) had a very high Mg content and hence a correspondingly low Ca. The actual Ca/P ratio in this would be a very misleading figure if not taken in conjunction with the corrected ratios. In passing to the rodents' teeth the results showed wide differences from those of the dentines. These teeth in general had a much greater Mg content and consequently a lower Ca content. Toverud [1923] found only 0·63 % Mg in rats' incisors, but Watchorn (private communication) found 1·6 % Mg in 4½-month-old rats on a diet containing 0·045 % Mg. In the present work values are high, no doubt because the diet was unintentionally but not abnormally high in Mg, i.e. 0·15 %. The proportion of Ca:Mg in these different materials shows that there are variations in the degree of replacement. In some cases (human and dog dentine) the molar relation Ca:Mg was approximately 18:1, in rodents' incisors 9:1, whilst in the elephant ivory it was 5:1 approximately. The difference in Mg content cannot altogether be accounted for by variations in Mg content of the blood sera. Human, dog, rabbit and rat sera contain respectively 2·2 [Greenberg & Mackey, 1932], 2·4 [Greenberg & Mackey, 1932], 2·6 [Brookfield, 1933], and 5·0 [Watchorn, 1933], all stated as mg. Mg/100 ml. The P values in rats' teeth were also significantly higher. The resulting Ca/P ratios were also much too low to satisfy $3Ca_3(PO_4)_2.MgX_2$. It is probable that Mg replaces even more of the Ca. A consideration of the "total" Ca and P values in comparison with the corresponding values in human dentine, etc. suggests that some other P compound, less basic than tricalcium phosphate, is present in rodents' teeth or that they contain considerable quantities of other bases not estimated. This still does not explain why the P content of the rodents' teeth was so much greater. X-ray analysis of rats' teeth and elephant tusk is being carried out in conjunction with this work and should help to settle the question of constitution.

A notable point is the difference in composition of the incisor and molar teeth of the same animal. The high Mg and low Ca/P of rats' incisors prompted this investigation on rabbits' and guinea-pigs' teeth. The rabbit and hare incisors corresponded reasonably well with rats' incisors. It was surprising to find much lower Mg values in guinea-pigs, but this was in agreement with the results of Toverud [1923] who found 1·74 % Mg. The difference between the incisors and molars in some cases is very marked and it does not seem possible to explain the differences by reason of the different proportions of enamel and dentine in the different teeth. The variations in Mg content of the rats' incisors at different ages agreed with the observations of Orent *et al.* [1934].

It would seem from the differences in composition of incisor and molar teeth of certain rodents and from the fact that the enamel and dentine which are laid down, presumably at the same time, in human teeth, can show distinct differences in Ca:Mg:P proportions, that the physico-chemical conditions which according to the supersaturation precipitation theory govern the deposition must be subject to local variations and not be determined by the blood system only. It may be noted in passing that though the Ca contents of different animals' blood do not differ very much, the P contents show big differences; for example pigs' blood contains much more inorganic P and ester-phosphorus than the blood of most other animals [Kay, 1928].

The fact that the proportion of Ca:Mg varies with age has also to be accounted for. Day *et al.* [1935] have dealt with this variation of Mg content of bone. It is possible that these age changes could be correlated with the recognized differences in blood composition at different ages [Watchorn, 1933], but it is not so easy to correlate the differences between incisor and molar teeth and bone taken at the same time, or the difference between enamel and dentine in human teeth. In the ash of human enamel the Ca/P is 2·15 and Mg 0·46 %, in the ash of dentine the Ca/P is 2·05 and Mg 1·2 %. The fact that big differences in mineral content can occur in material deposited in different regions of the body at the same time promotes the suggestion that all calcifications are the result of specific cell activity rather than merely precipitation governed by solubility products. Logan [1935] observed similar differences in composition of the inorganic part of calcified tissues and is of the opinion that these differences must be taken into account in any consideration of the means by which calcification takes place. There is evidence that the deposition of salts in bone, designated by Robison *et al.* [1930] the "inorganic" or second process in calcification (the hydrolysis of phosphoric esters by phosphatase apparently precedes this), is not a purely passive physico-chemical process because of the fact that *in vitro* it is inhibited by various means, partially by 0·001 *M* KCN, profoundly by 0·00001 *M* NaF [Robison & Rosenheim, 1934]. It is usual to associate such inhibitions with the cell activity. The last mentioned authors brought forward strong evidence that the second mechanism was also enzymic. For several reasons then calcification might be considered similar to other active and specific cell processes. The ameloblasts, odontoblasts and osteoblasts each, by the various enzymic processes, are responsible for laying down in an organic matrix complex phosphates of calcium and magnesium. The complexes are not necessarily universally identical but are affected by local conditions as well as by conditions in the blood. The organic composition of the matrix may be important in connexion with the variations in composition of the inorganic substance laid down therein. Harris [1932] found glycogen in developing bone. With the exception of liver cells in which glycogen represents a stored secretion, glycogen is usually regarded as a ready store of energy which is made available for cellular activity by formation of various esters of phosphoric acid. The glycogen of developing bone could play a similar role in the active cell processes. That glycogen metabolism may play a part in calcification is indicated by the fact that sodium iodoacetate in very low concentration inhibits the calcifying process *in vitro* [Robison and Rosenheim, 1934] and *in vivo* [Laszt & Verzár, 1935].

The theory built up by Robison from his extensive work on the calcifying mechanism has emphasized the sensitive character of the second mechanism. The results put forward here suggest that the calcification process is highly specific and that the differences in composition of the inorganic substance laid down in calcifying tissues are to be explained on the basis of this specificity.

SUMMARY.

1. A quantitative study of the Ca, P and Mg contents of the ash of teeth of different animals has been made. Marked differences were found in the relative amounts of these elements present.

2. Rodents' teeth generally contained more Mg, more P, but less Ca than dog and human teeth. Rodents' incisors and molars were different in composition.

3. Ca/P ratios varied between 1·75 (rats' incisors) and 2·09 (human dentine). This variation was due chiefly to differences in the Ca content resulting from the differences in Mg content. Ca/P ratios give no idea of the type of phosphate present. "Corrected" Ca/P ratios (see text) differed less.

4. The suggestion is made that the wide differences in composition of the mineral constituents found in teeth of different animals, in different types of teeth of the same animal, in bones and teeth of the same animal and in different tissues in the same tooth, support the view that the deposition process of calcification is an active and specific cell process, and not merely a precipitation from saturated or supersaturated solutions of a salt of constant composition dependent on the ionic composition of the blood plasma.

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CCXXI. THE ERROR DUE TO BLOOD ARGINASE IN THE UREASE METHOD FOR THE DETERMINATION OF BLOOD UREA.

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The possibility of an error in the determination of urea in blood by urease methods was brought to our notice by the fact that figures obtained by the direct estimation of urea in human blood corpuscles were obviously too high. This error appears to have been first reported by Behre [1923], who observed that when the soya bean urease method was applied to whole blood the urea obtained was partially determined by the concentration of urease extract employed, and that the substance responsible for this "extra urea" was chiefly contained in the corpuscles. She did not come to any conclusion as to the exact nature of this substance. Addis [1928], when estimating the urea in liver tissue with Jack bean urease, obtained a linear relation between the quantity of urea produced and the quantity of urease used. He considered that the extra urea was derived from the action of arginase in the liver on arginine in the Jack bean. He also found that the apparent concentration of urea in human blood might be varied by adding different amounts of urease. He does not state that the variation in the case of blood was due to arginase. Peters & Van Slyke [1932], in describing the urease method as applied directly to whole blood, state "...unnecessarily prolonged contact between blood and enzyme is to be avoided because of the possibility of slow formation of ammonia by the action of arginase in the blood cells on arginine in the enzyme preparation" and refer to the work of Addis and Behre which did not actually prove that the error was due to the action of blood arginase.

This source of error in a standard method appeared to be of sufficient importance to justify a further investigation.

EXPERIMENTAL.

Method. Urea was determined by the aeration-titration method as described by Kay and Sheehan [1934] except that the acid was titrated to the nearest 0·05 ml. in a standard 50 ml. burette instead of to 0·01 ml. as in the original method. All determinations were done in duplicate and the figures for blood are given to the nearest 0·5 mg. urea per 100 ml. blood.

Urease. 1. A watery extract of soya bean powder prepared by extracting 10 g. of powder with 100 ml. of water for 1 hour as described by Kay & Sheehan [1934]. 2. Jack bean urease powder, acetone precipitate [Peters & Van Slyke, 1932]. 3. Crystalline urease prepared by the method of Sumner [1926, 1, 2]. It was found that good crystals could be obtained by using Jack Bean Powder (B.D.H.), which had been thoroughly extracted with ether in a Soxhlet apparatus and then dried, in place of the "Arlco" Jack bean meal used by Sumner.

Blood. Blood was taken from a limited number of normal men by venipuncture and oxalated in the usual manner.

Preliminary experiments confirmed the findings of Behre and Addis that extra urea was formed when the concentration of enzyme was increased. The action of a strong solution of urease was then compared with that of a preparation of crystalline urease on blood and plasma simultaneously. It will be seen from Table I (Nos. 1–4) that the 100 mg. of powder gave 6·5 mg. of urea more than the crystalline enzyme with whole blood, but that the two preparations gave identical results with plasma.

Table I.

Subject	Substrate	Urease preparation	Urea mg. per 100 ml. blood or plasma
1. S. L. T.	Blood	100 mg. powder	25·5
2. ,,	Blood	Crystalline	19
3. ,,	Plasma	100 mg. powder	23
4. ,,	Plasma	Crystalline	23
5 A. D.	Blood inactivated	Soya bean extract (5 ml.)	25
6. ,,	Blood inactivated + 2 mg. arginine	,,	25
7. ,,	Blood	,,	30
8. ,,	Blood + 2 mg. arginine	,,	39·5
9. A. B. A.	Blood inactivated	,,	19·5
10. ,,	Blood inactivated + 2 mg. arginine	,,	20·5
11. ,,	Blood	,,	31
12. ,,	Blood + 2 mg. arginine	,,	34
13. R.	Plasma inactivated	,,	29·5
14. ,,	Plasma inactivated + 2 mg. arginine	,,	28·5
15. ,,	Plasma	,,	29·5
16. ,,	Plasma + 2 mg. arginine	,,	29·5
17. T. P.	Blood	100 mg. powder	24·5
18. ,,	Blood + 2 mg. $CoCl_2$, $6H_2O$	,,	49·5
19. ,,	Blood inactivated	,,	21·5
20. ,,	Blood inactivated + 2 mg. $CoCl_2$, $6H_2O$	,,	41·5
21. S. L. T.	Blood	100 mg. powder	32
22. ,,	Blood + 2 mg. $CoCl_2$, $6H_2O$	,,	60·5
23. ,,	Plasma	,,	30·5
24. ,,	Plasma + 2 mg. $CoCl_2$, $6H_2O$	,,	32·5
25. A. B. A.	Blood	,,	37·5
26. ,,	Blood + 2 mg. glutamic acid, 2 mg. histidine hydrochloride, 2 mg. glycine	,,	38·5
27. ,,	Blood + 2 mg. arginine	,,	49·5

It was then assumed that these results were due to the action of blood arginase. The addition of arginine in solution to the blood was found to increase the apparent urea. Now Hunter and Dauphinee [1933] have shown that arginase is rapidly inactivated at a *p*H less than 4·0. Accordingly blood arginase was inactivated in the following manner: to 2 ml. of blood washed into the reaction tube with water instead of buffer was added 1 ml. of N H_2SO_4 and the tube heated to 50° for 10 min., the tube was then cooled and the acid neutralized by 1 ml. of N NaOH and then the buffer and urease added. An equivalent amount of a mixture of equal volumes of N H_2SO_4 and N NaOH was added to the other tubes in the series. Not only was no extra urea produced by the addition of arginine to the "inactivated" blood but the apparent urea in the "inactivated" blood was less than in the untreated blood, while the addition of arginine to the untreated blood produced a considerable increase in the urea found. (Table I, Nos. 5–8.) Blood from a different individual showed the same effect except that there was a very slight increase of urea on adding arginine to the "inactivated" blood. (Table I, Nos. 9–12.) On the other hand the values for plasma urea were unaffected by this "inactivation" nor did the plasma give any extra urea on the addition of arginine. (Table I, Nos. 13–16.)

In a recent paper Hellerman and Perkins [1935] demonstrated the activation of arginase by certain metal ions Co^{++}, Ni^{++}, Mn^{++}. The effect of the addition of a solution of cobalt chloride to the reaction tubes was therefore investigated. Cobalt chloride 2 mg. in 2 ml. of water produced an increase of approximately 100% in the apparent urea of the blood. (Table I, Nos. 17, 18.) This extra 25 mg. of urea per 100 ml. of blood would be equivalent to the hydrolysis of 1·45 mg. of arginine in the reaction tube. The "inactivated" blood was also reactivated by the cobaltous ions and gave an extra 20 mg. of urea. (Table I, Nos. 19, 20.) With blood and plasma the addition of cobalt again produced a large increase in the urea found in the blood and only a very small increase with plasma which was probably due to a small amount of corpuscle arginase contaminating the plasma as there was slight haemolysis. (Table I, Nos. 21–4.) As a control tubes were put up without blood. Both urease with cobalt and also urease with arginase and cobalt failed to form ammonia. The titrations were identical with that for the urease blank.

Other amino-acids were tried in place of arginine. In one experiment glycine gave no extra urea and in another a mixture containing glutamic acid 2 mg., histidine hydrochloride 2 mg. and glycine 2 mg. produced only the slight increase of 1 mg. per 100 ml. in the apparent urea, while the addition of 2 mg. of arginine gave an increase of 12 mg. (Table I, Nos. 25–7.)

Urease solutions were incubated with a preparation of liver arginase—made from calf liver by the method described by Hellerman & Perkins [1935]—instead of blood, and it was found that the arginase produced ammonia from the strong urease solutions. It will be seen from Table II that 10 mg. of urease powder incubated with arginase gave no appreciable ammonia though the arginase solution was active and hydrolysed added arginine. When the arginase was activated by cobalt a small quantity was produced. By contrast 100 mg. of urease powder, though only giving a small quantity of ammonia with arginase alone, with cobalt-activated arginase gave a large amount of ammonia equivalent in this case to the hydrolysis of 1·85 mg. of arginine. The quantities of ammonia produced from this strong urease solution are of the same order as those equivalent to the "extra" urea obtained with blood under similar conditions.

Table II. *The reaction between arginase and urease preparations.*

	ml. 0·01 N H_2SO_4 equivalent to NH_3 formed after incubation at 50° for 30 min. with urease powder	
	10 mg.	100 mg.
1. Arginase solution 2 ml.	0·0	0·35
2. Arginase solution 2 ml. + arginine 2 mg.	0·9	0·7
3. Arginase solution 2 ml. + $CoCl_2, 6H_2O$ 2 mg.	0·3	2·15

Discussion.

The experiments reported here show that the factor responsible for the production of the extra urea from the urease solutions is the arginase in the corpuscles, because it is activated and inactivated by the same methods as arginase and can be replaced by a preparation of arginase from liver. Addis, in his experiment with liver and urease, considered that it was unlikely that the arginine in some of his urease preparations was free, and suggested that either proteolytic enzymes in the liver set arginine free, or that one of the proteins of the Jack bean has arginine so situated in its structure that urea might be formed from it by arginase. We have no direct analytical evidence of the amount of free arginine in our urease preparations but free arginine cannot be excluded on general grounds. Arginine is precipitated by acetone in the strength used for precipitating the soluble urease powder and according to Sasaki [1932] arginine nitrate 9·55 g. was isolated from the hot water extract from 5 kg. soya bean which had previously been extracted with ether.

According to Edlbacher *et al.* [1927] arginase is present in the blood of man, cattle, sheep and pig, and absent from the blood of the dog, cat, rabbit, porpoise, mouse, rat, hen, pigeon and goose [Edlbacher & Rothler, 1925]. On the other hand Weil & Russell [1934] report the presence of arginase in rat blood in both the cells and plasma and that it is higher in adult males than in young males, castrates and females, but there is no obvious relationship between the blood urea level and the arginase activity. There seems to be general agreement that arginase is present in human blood and that it is confined to the cells.

In the experiments reported above the reaction mixtures were all incubated for 30 min. The effect of the time of incubation has not been investigated in detail but bloods from some subjects were found to give appreciable amounts of extra urea after only 8 min. incubation. The original observations of Behre were made after incubation for 10 min. on bloods obtained from hospital cases which showed considerable variation in the magnitude of the increase in urea with concentrated extracts. It is of interest to note that even with the weak extracts used by Behre [1923] the figure for whole blood urea was in some cases a little higher than that for plasma indicating a small production of extra urea. The urease preparations used in the present work though described as strong solutions were actually of the strength advised for routine use.

It seems unlikely that this error is of significance in the routine estimation of blood urea for clinical purposes. When however an accurate estimation is necessary the variable error introduced by the action of blood arginase may be far greater than any experimental error and will vitiate the result unless precautions are taken to eliminate the arginase action. The question would not arise with an animal such as the rabbit whose blood is said to be free from arginase. In the case of human blood the most satisfactory solution appears to be the use of plasma for analysis. In the presence of cobaltous or other divalent ions which activate arginase such precautions would be essential.

SUMMARY.

1. The reported production of "extra" urea when human blood is incubated with concentrated solutions of urease is confirmed.

2. The substance responsible is considered to be the blood arginase. It is confined to the corpuscles and is inactivated by incubation at 37–50° for 10 min. after the addition of acid to the blood and is activated by the addition of cobaltous ions which may produce an increase of 100 % in the apparent blood urea. A similar production of ammonia is observed when the concentrated urease solutions are incubated with a preparation of liver arginase.

3. The significance of this error in urea analysis is discussed and the use of plasma is advised for the accurate estimation of urea in human blood.

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CCXXII. RESPIRATION OF BRAIN.

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(*Received 13 March 1936.*)

Few facts are known concerning the metabolism of parts of the brain other than cerebral cortex. Holmes [1930] has shown that grey matter is more active than white in its oxidising systems. Respiration rates of roughly differentiated parts of the brain in Ringer-phosphate-glucose have been studied by Gavrilescu & Peters [1931] in the pigeon and by Himwich & Fazikas [1932] in the rat. Experiments have been started in this laboratory to extend further this differentiation of brain regions by studying respiration rates with different substrates, and parts have been selected which are known to be especially susceptible to pathological change. Thus we have chosen the striate body, globus pallidus, cerebellar cortex and the cornu ammonis. We have also included the hypothalamus and medial parts of the thalamus because of their alleged specific affinity for narcotics, particularly the barbiturates.

The present investigation deals with the metabolism of these parts in normal ox brain.

Experimental.

Respiration rates were measured in Barcroft differential manometers at 37·5°. The medium was Ringer-phosphate as used by Gavrilescu & Peters [1931]. The manometers were filled with oxygen. The ox brains were received about 1 hour after death of the animal and the first manometer readings were normally taken about 2 hours after death. After removal of the membranes, slices of the various parts were cut with a small arrow-shaped scalpel and were then either transferred to Ringer solution or mashed for about 30 sec. on a glass plate with a bone spatula. Slices of cerebral cortex about 1 cm. square were easily cut by this method, but from cerebellar cortex only thin strips could be cut owing to its structure. About 4 or 5 such strips, usually cut from folds of the vermis, were used in experiments with slices. The cornu ammonis and corpus striatum (caudate nucleus) were minced or sliced after removal of the ependyma. The thalamus with the hypothalamus and corpus callosum were easily dissected out *en bloc* for mincing or slicing. The globus pallidus is so small and its respiration so low that it was necessary to pool tissue from six ox brains for a satisfactory experiment. Normally about 120 mg. tissue were used.

Table I and Fig. 1 summarize the results obtained on minced tissue from different brain regions with or without the addition of glucose, lactate, succinate etc., in Ringer-phosphate solution.

It is seen that minced cornu ammonis and globus pallidus show no extra oxygen uptake in presence of glucose and little in presence of lactate, although these parts show an increased respiration in presence of succinate. The respiration of cerebellar cortex, corpus striatum and thalamus in glucose is also affected

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[2] Under a grant from the Rockefeller Foundation.

Table I. *Effect of substrate on oxygen uptake of minced brain tissues.*

μl. O_2/g. fresh tissue/hour.

		Cerebral cortex			Cerebellar cortex			Thalamus and hypothalamus			Corpus striatum		
Exp.	Substrate	1 hr.	2 hr.	3 hr.	1 hr.	2 hr.	3 hr.	1 hr.	2 hr.	3 hr.	1 hr.	2 hr.	3 hr.
1	None	702	500	368	936	507	352	574	483	374	806	594	440
2	Glucose	876	811	750	1010	783	622	—	—	—	1000	829	692
3	,,	898	878	820	—	—	—	602	520	430	919	827	624
4	Lactate	939	848	700	1145	870	680	—	—	—	1011	788	568
5	,,	1295	1136	1008	1708	1360	1092	—	—	—	—	—	—
6	,,	920	848	752	—	—	—	863	664	537	1158	841	639
7	Succinate	1740	1130	745	1833	1117	706	—	—	—	2092	1316	800
8	,,	—	—	—	2065	1400	960	—	—	—	2049	1600	1112
9	,,	1910	1380	970	—	—	—	1340	1040	800	2365	1600	970
10	Lactate-glycero-phosphate	1450	1070	815	—	—	—	1160	880	625	1670	1080	680
11	,,	1620	1140	—	2080	1480	—	—	—	—	1760	1460	—
12	Glutamate	806	622	540	880	568	392	658	512	416	890	636	492

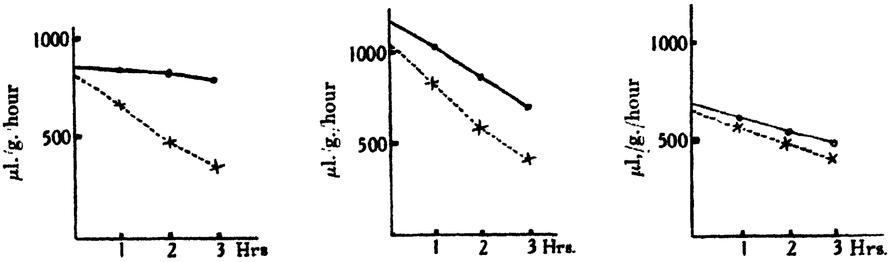
		Cerebral cortex			Other brain region		
Exp.	Substrate	1 hr.	2 hr.	3 hr.	1 hr.	2 hr.	3 hr.
						Corpus striatum	
16	None	705	528	408	667	532	408
	Glucose	767	711	650	740	608	464
						Corpus callosum	
17	None	761	524	390	100	66	60
	Glucose	880	824	750	100	64	55
						Corpus striatum	
32	None	829	692	500	679	556	412
	Na butyrate	692	538	400	487	360	256
						Cerebellar cortex	
34	None	692	476	282	933	500	344
	Na glutamate	816	624	560	936	520	372
35	None	672	488	380	812	372	280
	Na glutamate	776	628	520	872	540	360

		None			Glucose			Lactate		
Exp.	Brain region	1 hr.	2 hr.	3 hr.	1 hr.	2 hr.	3 hr.	1 hr.	2 hr.	3 hr.
21	Cornu ammonis	543	394	268	550	334	228	678	454	280
25	Globus pallidus	228	138	96	206	140	90	250	172	104
30	Cerebellar cortex	980	550	380	1100	880	670	1680	1340	1010

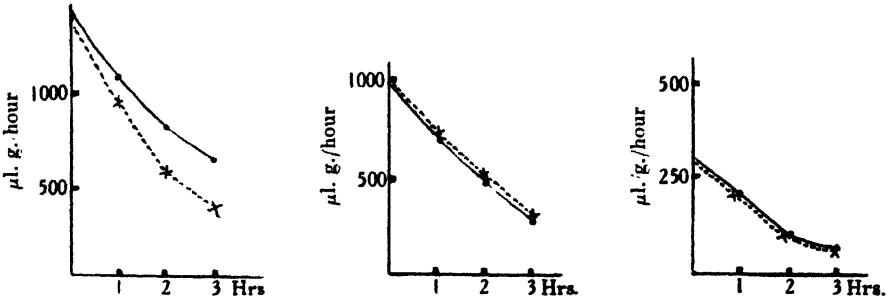
though to a less extent. It is of course well known that most tissues have very powerful succinoxidase systems which can be freed from cell débris [compare Cohen & Gerard, 1934].

It was tempting to ascribe these results to real differences in the biochemistry of these regions. When, however, slices of these parts were used a constant respiration rate was always obtained, in glucose or lactate, and the usual falling off of oxygen uptake in absence of added substrates. Table II summarizes the results obtained for the respiration of sliced tissue in Ringer-phosphate with glucose or lactate.

The oxygen uptake values given in Table II were roughly constant during the 3-hour period of the experiment.



Exp. 18. Cerebral cortex. Exp. 19. Striate body. Exp. 29. Thalmus + hypothalamus.



Exp. 22. Cerebellar cortex. Exp. 23. Cornu ammonis. Exp. 23. Globus pallidus.

Fig. 1. •—• Ringer-phosphate + glucose; ×----× Ringer-phosphate.

In the absence of added substrate the oxygen uptake of each part decreased rapidly from an initial value which was roughly the same as that with glucose to about 1/3 of this in 1½ hours. The slices of tissue were removed from the bottles and the dry weights found in the usual way. The dry weight of cerebral and cerebellar cortex is about 17 % of the wet weight and those of corpus striatum, thalamus and globus pallidus, about 18 %. These ratios have been used to calculate the oxygen uptakes in µl./g. wet weight/hour from the dry weight.

Table II. *Oxygen uptakes of sliced tissue in µl./g. wet weight/hour.*

	Glucose	Lactate
Cerebral cortex	1700	2040
Cerebellar cortex	2550	3060
Corpus striatum	1980	2160
Cornu ammonis	1260	1440
Thalamus	1170	1440
Globus pallidus	360	450

Further experiments were carried out in which about 30 slices of cerebral cortex were cut from a single fresh ox brain. Half of these slices were then minced in the usual way and the respiration of the mince so prepared was compared with that of sliced tissue unminced. About 100 mg. of tissue were used for each Barcroft bottle. It is easy to obtain duplicate respiration rates of minced tissue agreeing within 5 % with this amount of tissue, but results on single cortex slices may vary by 20–30 %. To obtain good agreement of duplicates in the slicing series, three or four representative slices were used for each bottle and duplicates then agreed to within 10 %. One of three such experiments is recorded here.

Medium: Ringer-phosphate + glucose 0·2 %.

Oxygen uptake in µl. per g. wet weight over 2-hour period during which respiration rate of tissue did not change appreciably:

Minced	1600
Sliced	4000

In the case of cerebral cortex then, the respiration of the slice (2000 µl./g./hour) is about double that of the mince (800 µl./g./hour) and this difference between sliced and minced tissues is even more marked in other parts of the nervous system. This does not agree with the work of Holmes [1930] and of Quastel & Wheatley [1932] who state that mincing the brain tissue does not alter the respiration rate.

As the Ringer-phosphate solution used in the above experiments contained a rather large amount of potassium it was considered that this factor might possibly account for these results, since Dickens & Greville [1935] have shown that *M*/10 KCl added to Ringer solution increases the respiration of rat cortex slices by about 100 % in glucose or lactate. It was found that such an addition of potassium to *M*/60 sodium phosphate-Ringer produced a slight inhibition of the respiration of minced ox cortex or minced rat whole brain. The respiration of ox cortex slices was increased about 30 % in glucose as against 100 % in the rat. The difference between the mince and the slice was therefore not due to the large potassium content of the medium.

Oxygen uptake of cerebral cortex in Ringer-phosphate-glucose with and without M/10 KCl in µl./g. wet weight/hour.

	Without KCl	With KCl
Minced	850	800
Sliced	1700	2380 (KCl added after 40 min.)

Duration of experiment 80 min. 0·3 ml. of *M* KCl contained in "danglers" and added to the 3 ml. of medium in the Barcroft cups by shaking after 40 min. Medium: NaCl 0·70 %, KCl 0·018 %, $CaCl_2$ 0·019 %, NaH_2PO_4 0·04 %, Na_2HPO_4 0·21 %, as used by Warburg [1923].

Differences in the magnitudes of the respiration rates of the various parts are not necessarily indicative of relative cellular metabolic activities, since it is not known how much is due to variation in cell density. It is however interesting to note that the respiration rate of cerebellar cortex is initially about 50 % higher than that of cerebral cortex. In the minced tissue experiments, diminution of the respiration rate was most marked in those regions which contain specially large ganglion cells like the cornu ammonis and the cerebellar cortex. It was therefore supposed that these large ganglion cells suffered more damage on mincing. Confirmation of this was obtained by histological examination of films of minced tissue stained with toluidine blue, since the large pyramidal cells of the cornu ammonis and the Purkinje cells of the cerebellum were often nearly completely destroyed, whereas the smaller cells of the cerebral cortex preserved their shape better. Examination of slices of tissue revealed no such damage to the large ganglion cells. This explanation then may account for some of the differences observed in the minced tissue experiments, although it must be pointed out that the large ganglion cells in the cerebellar cortex and cornu ammonis constitute a fraction of the total ganglion cell content of these regions which is difficult to assess since there are both large and small ganglion cells. The same applies to the thalamus, the hypothalamus and the striate body, whilst the globus pallidus contains only large cells which however do not attain the size of the Purkinje cell.

It was then considered possible that injury to the large cells of the gasserian ganglion might have similarly damaged their respiration in the experiments of Holmes [1932] who, using chopped tissue, found that their respiration was very low in glucose, being actually less than that of the cranial nerve. On the basis of these experiments Holmes suggested that the seat of metabolic activity is the dendrite rather than the cell body, since the gasserian ganglion cells have no dendritic structures. The point was therefore investigated in the ox by removing the two ganglia and cutting slices of them with a razor or chopping them with scissors. Simultaneously the brain was removed and slices of cerebral cortex and cerebral white matter used for comparison. It was found that even the slight injury to the ganglion resulting from chopping with scissors caused the respiration rate in glucose to fall rapidly to one-half of its initial value in 2 hours. The respiration of the sliced ganglion, on the other hand, was not only much higher than that of the minced ganglion or of sliced white matter, but was constant over the 2-hour period of the experiment in glucose and fell rapidly in its absence, resembling that of cerebral cortex in this respect. Although the magnitude of the oxygen uptake of the sliced ganglion is low (150 µl./g./hour) when compared with that of cortex (1700µl./g./hour) yet it must be concluded that in the ox the cells of the gasserian ganglion oxidize glucose, even though they have no dendritic structures, and that they are more efficient in this respect than white matter.

Oxygen uptakes of gasserian ganglion and corpus callosum in µl./g. wet weight/hour.

		Sliced	
Minced		With glucose	Without glucose
Gasserian ganglion	100	300	90
White matter (corpus callosum)	60	180	70

The above figures represent average oxygen uptakes over the 2-hour period of the experiment. Medium: Warburg's [1923] Ringer-phosphate + glucose.

The steady maintenance of the respiration rate of mammalian brain when minced is a point often cited when the metabolism of brain is compared with that of other tissues. The respiration of the liver for example is almost completely destroyed by mincing. The experiments here recorded show that even if the respiration of cerebral cortex appears to be undamaged by mincing it is in reality injured and the injury to other parts is more marked. As a whole brain tissue may be less damaged than, say, liver tissue, not because of any essential biochemical difference but because it is less susceptible to mechanical injury. The above investigation illustrates the necessity for accurate morphological control in the interpretation of biochemical findings.

SUMMARY.

Respiration rates of different parts of the brain which are known to be especially vulnerable have been studied in the presence of different substrates. In the minced tissue, unlike cerebral cortex, certain regions showed little or no extra oxygen uptake in the presence of glucose or lactate, whereas, when slices of these regions were used, constant and much higher respiration rates were observed. Cerebellar cortex has a greater metabolic activity than any other part of the brain.

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CCXXIII. HYPOGLYCAEMIC AND OTHER ACTIONS OF PHOSPHOTUNGSTIC ACID, PHOSPHOMOLYBDIC ACID AND ALLIED SUBSTANCES.

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On account of their chemical properties phosphotungstates and phosphomolybdates have been long used in the laboratory for colorimetric estimations but their actions on the living system have not been properly studied. Administered internally these substances, however, were found to have definite effects on metabolism. In a previous communication [Mukherjee, 1935] it was shown that phospho-24-tungstic acid had hypoglycaemic action. As mentioned in that paper experiments were also done with some allied substances. It was found from these experiments that phosphomolybdic acid, molybdic acid, silicotungstic acid, sodium tungstate, ammonium phospho-18-tungstate and also sodium vanadate had hypoglycaemic actions when administered by mouth. Some of these substances were found to have diuretic actions as well. It was also found from experiments that these substances acted like oxidizing enzymes. From a consideration of these properties the author was led to try the effect of these substances in a few cases of cancer [Mukherjee, 1936]. This work is being continued. In the present paper the hypoglycaemic and other actions of these substances will be dealt with.

EXPERIMENTAL.

Excepting phosphotungstic and silicotungstic acids (Schering-Kahlbaum) the other substances used were Merck's. Ammonium phospho-18-tungstate (Wu's B form) was prepared in the laboratory [Wu, 1920].

Hypoglycaemic action. For studying the hypoglycaemic action white rabbits were used. In a few cases experiments were also done on diabetic human subjects. Blood sugar was estimated by the Folin-Wu colorimetric method. The hypoglycaemic effect was measured by the fall of blood sugar 3 hours and also 5 hours after administration of the material in rabbits fasting for about 20 hours. The samples were administered in 5 ml. of distilled water by stomach tube to the

Table I.

			Insulin 0·6 unit subcutaneously. Blood sugar in mg. per 100 ml.			Phosphotungstic acid (0·3 g.). Blood sugar in mg. per 100 ml.			Phosphomolybdic acid (0·3 g.). Blood sugar in mg. per 100 ml.		
Rabbits	Wt. kg.	Sex	Initial	3 hr.	5 hr.	Initial	3 hr.	5 hr.	Initial	3 hr.	5 hr.
I	1·26	F	105	67	80	102	71	67	89	77	74
II	1·16	F	91	63	65	87	71	71	87	80	63
III	1·22	M	100	74	—	83	67	—	95	83	80
IV	1·20	M	80	59	—	91	77	74	83	74	71
V	1·31	F	111	67	91	118	87	83	111	85	83
VI	1·14	F	91	77	80	95	91	87	100	83	83

rabbits, usually at intervals of 2–3 days. The hypoglycaemic effects of 0·3 g. of phosphotungstic acid and 0·3 g. of phosphomolybdic acid compared with 0·6 unit of insulin (Eli Lilly and Co.), 0·5 unit per kg. body weight, given subcutaneously are shown in Table I.

The effects by mouth of 0·3 g. of molybdic acid and 0·3 g. of sodium tungstate compared with 0·7 unit of insulin, 0·5 unit per kg. body weight, given subcutaneously are shown in Table II.

Table II.

			Insulin 0·7 unit subcutaneously. Blood sugar in mg. per 100 ml.			Molybdic acid (0·3 g.). Blood sugar in mg. per 100 ml.			Sodium tungstate (0·3 g.). Blood sugar in mg. per 100 ml.		
Rabbits	Wt. kg.	Sex	Initial	3 hr.	5 hr.	Initial	3 hr.	5 hr.	Initial	3 hr.	5 hr.
VII	1·43	F	105	74	91	105	91	91	100	95	87
VIII	1·22	F	91	67	74	95	83	80	105	74	74
IX	1·56	M	95	80	—	105	80	77	95	91	80
X	1·60	M	100	67	91	91	77	74	—	—	—

The hypoglycaemic effects by mouth of 0·2 g. of sodium vanadate (meta) and of 0·3 g. of silicotungstic acid and 0·3 g. of ammonium phospho-18-tungstate were also similarly determined (Table III).

Table III.

	Sodium vanadate (meta) (0·2 g.). Blood sugar in mg. per 100 ml.			Silicotungstic acid (0·3 g.). Blood sugar in mg. per 100 ml.			Ammonium phospho-18-tungstate (0·3 g.). Blood sugar in mg. per 100 ml.		
Rabbits*	Initial	3 hr.	5 hr.	Initial	3 hr.	5 hr.	Initial	3 hr.	5 hr.
I	95	80	77	—		—	—	—	
II	91	74	69	—	—	—	—	—	—
VII	100	74	74			—	—	—	—
VIII	91	67	—	—	—	—	—		—
V	—	—	—	—	—	—	102	74	77
VI	—	—	—	83	71	67	95	67	67
IX		—	—	87	77	71	87	83	74
X	—	—	—	111	74	77	—	—	—

* The same rabbits as in Tables I and II.

As a control test the rabbits were given 5 ml. of distilled water each by stomach tube. There was in this case only insignificant variation in blood sugar level (after 3 and 5 hours). In some rabbits there was rather a slight rise of blood sugar.

Diuretic action. While studying the hypoglycaemic effects of the substances it was observed that the rabbits micturated rather more frequently. The hypoglycaemic and diuretic effects by mouth of 0·9 g. of phosphotungstic acid in 30 ml. of distilled water were also determined in 4 diabetic patients. The material was administered by mouth to the fasting patient in the morning. The initial fasting blood sugar before administration of the material and blood sugar 3 hours after administration of material were estimated. The patients were also given the same dose of phosphotungstic acid (0·9 g. in 30 ml. of distilled water) in the evening and the diuretic effect was determined by measuring the 24 hours' urine volume before and after administration of material. The hypoglycaemic effect of 0·9 g. phosphotungstic acid by mouth in diabetics compared with that of 5·0 units of insulin given subcutaneously and the diuretic effect of 0·9 g. phosphotungstic acid given by mouth morning and evening are shown in Table IV.

Table IV.

				5·0 units of insulin subcutaneously. Blood sugar in mg. per 100 ml.		0·9 g. of phosphotungstic acid by mouth (morning and evening)			
						Blood sugar in mg. per 100 ml.		Urine volume in litres	
Diabetic patients*	Age	Sex	Wt. kg.	Initial (7 A.M.)	3 hr.	Initial (7 A.M.)	3 hr.	Before	After
A	42	M	37·4	286	182	286	200	1·94	2·46
D	40	M	51·0	220	67	182	87	1·51	2·22
R	50	M	52·0	268	133	250	160	1·90	2·62
M	45	M	42·4	167	65	191	95	1·68	2·10

* Breakfast given after 10 A.M.

Oxidizing action. It was found from experiments that these substances acted like oxidizing enzymes. Crystals of phosphomolybdic acid (about 0·1 g.) when dropped into a test-tube containing about 5 ml. of a solution of benzidine in acetic acid gave a blue colour to the solution even without addition of hydrogen peroxide. On addition of hydrogen peroxide the blue colour became deeper with evolution of oxygen. Crystals of phosphomolybdic acid (about 0·1 g.) when dropped into a test-tube containing about 5 ml. of "Merckozone" (Merck's hydrogen peroxide) caused evolution of oxygen.

With the benzidine reagent phosphomolybdic acid alone gave a definite positive reaction. With reduced alkaline phenolphthalein reagent [Johannessen, 1921; Stammers, 1926], however, phosphotungstic acid, silicotungstic acid, sodium tungstate, ammonium phospho-18-tungstate, phosphomolybdic acid, molybdic acid, ammonium molybdate, sodium vanadate in about 0·05 g. amounts all gave positive reactions (red colour).

DISCUSSION.

From the experiments it is evident that phosphotungstic acid, phosphomolybdic acid and many allied substances have definite effects on metabolism when administered internally in sufficient amounts. Vanadates also appear to have similar effects. When compared with insulin the hypoglycaemia given by these substances apparently runs a more prolonged course. These substances also act like oxidizing enzymes and the association of oxidizing action with hypoglycaemic action in the same substance suggests that the hypoglycaemia is perhaps due to oxidation of sugar in the tissues. Some of the substances were found to have diuretic actions also. These substances (vanadates excepted) were not found to have any toxic effect in the doses administered.

SUMMARY.

1. Phosphotungstic acid, phosphomolybdic acid and many allied substances (also vanadates) cause hypoglycaemia when administered by mouth in proper amounts (about 0·3 g. in rabbits).
2. These substances also act like oxidizing enzymes.
3. Some of these substances (e.g. phosphotungstic acid) have diuretic actions as well.

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CCXXIV. AN APPARATUS FOR DISTILLATION.

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An excellent means of distilling water and concentrated acids is afforded by the apparatus shown in the accompanying diagram.

The apparatus is constructed of pyrex glass throughout. A two-litre flat-bottomed flask, having a neck provided with a ground glass joint of standard size, is fitted with a still head of the pattern shown. Part of the latter is sloped in such a way as to ensure return to the flask of any spray which might possibly pass the trap. In actual practice, however, water, hydrochloric and nitric acids boil so evenly and gently in the large flask that there seems no possibility of any spray reaching the distillate. The other end of the still head slips into the condenser, and the cowl, which is part of the still head, is a protection against dust. No ground glass joint is necessary at this point, and this gives "flexibility" to the apparatus, so that the only support required is the tripod to carry the flask and a retort stand to hold the condenser. This "flexibility" also enables the apparatus to be assembled or dismantled in a few seconds without risk of fracture.

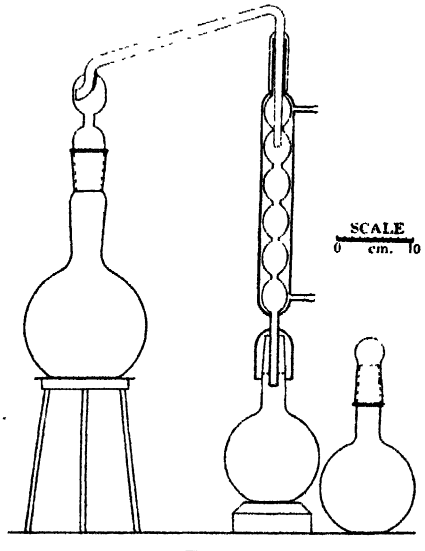

Fig. 1.

The condenser contains six bulbs. The upper two constrictions between the bulbs should be wide enough to allow the insertion of the tube of the still head to the distance shown, but the lower constrictions should be somewhat narrower to ensure the efficient condensation of the distillate. There is no tendency for the vapour to pass upwards. The product of the distillation may conveniently be collected in litre pyrex flasks of the form illustrated, and these should be provided with standard size external fitting caps as shown.

The apparatus herein described has been in use for a considerable time, being mainly employed for the distillation of water to be used for the preparation of colloidal gold solutions suitable for Lange tests, and has given excellent results with the minimum of trouble. The apparatus is also well suited for the preparation of nitric acid free from chlorides or metals, and the acid may be distilled as easily as water. Another use for the apparatus is the distillation of strong hydrochloric acid for the preparation of standard solutions [Peters & Van Slyke, 1932]. This excellent method of preparing standard acid of high accuracy is not widely used because of the difficulty of distilling such strong acids as 20 % hydrochloric. With the above apparatus, however, the preparation of standard acid is facilitated.

The apparatus may be purchased from R. B. Turner and Co., 9–11 Eagle Street, London, W.C. 1, or from Quickfit and Quartz Co. Ltd., Albemarle Street, London, W. 1.

REFERENCE.

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CCXXV. STUDIES OF THE HAEMOGLOBIN AND IRON OF THE BLOOD.

I. THE DETERMINATION OF THE TOTAL IRON OF BLOOD.

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(*Received 2 July 1936.*)

METHODS for the determination of the total iron content of blood have been the subject of very active investigation for many decades and the great advances in knowledge of all forms of anaemia during the past ten years have greatly stimulated these researches.

Gravimetric methods were evolved more than a century ago and gave good results, but they necessitated the use of large volumes of blood of the order of 100–200 ml. Attention was therefore turned to titrimetric methods in which the iron content of ash obtained by incinerating blood was estimated by titration against potassium permanganate, titanous solutions, or thiosulphate after the addition of potassium iodide.

About forty years ago the advantages of colorimetric methods were being realized. It was found that the iron of the ash of blood could be estimated by examination of the Prussian Blue colour developed when ferrocyanides are added, or of the red colour formed on the addition of thiocyanates, under suitable conditions, to the ash. The latter colour reaction has formed the basis of almost all the work of the past thirty or forty years, but many workers have thought that it does not give correct results, especially with regard to tissues rich in phosphorus.

Bogniard & Whipple [1932], Klumpp [1934], Horwitt [1934] and others have therefore revived methods using titanous salts to estimate, by titration, the iron content of ashed material, but the shortcomings of these methods have been pointed out by them and others. Titanous salts react not only with iron salts but also with those of copper, tin, platinum and mercury, so that results in the presence of any of these other elements will give too high a value for the iron content. Moreover, when the end-point is determined colorimetrically, the presence of phosphates tends to obscure this point. The chief difficulty, however, is the necessity of preserving the titanous solution under carbon dioxide, hydrogen, or some other inert gas and of carrying out the titration in an inert atmosphere. In addition, the solutions must be frequently standardized.

Efforts have recently been made to devise, with other reagents, colorimetric methods free from the disadvantages of the thiocyanate reaction. Burmester [1935] and Tompsett [1934] have used thiolacetic acid.

Hill [1931] has shown that dipyridyl, in the presence of small quantities of iron, gives an extremely stable pink colour which is not affected by a wide variation in conditions. This marked specificity for iron, together with other useful properties, has quickly given dipyridyl a definite place in iron analysis and several workers have adapted it for various purposes. Elvehjem *et al.* [1933] have used it for estimating the "available" iron of wheat, yeast etc., as have also Shackleton & McCance [1936]; Engel [1934] has employed it in determining the iron in the enamel of teeth, and Cooper [1935] for the iron in the sea and in marine plankton.

This communication describes a method of estimating the total iron content of blood by the use of dipyridyl.

REAGENTS.

1. *Approximately 50% sulphuric acid.* Dilute a convenient quantity of concentrated sulphuric acid to twice its volume with distilled water. It is often not necessary to distil the strong sulphuric acid because so little of the reagent is used for each estimation and most A.R. samples show only the slightest traces of iron. For the purposes of this paper, however, all sulphuric acid was distilled.

2. *Concentrated nitric acid.* As this acid is used in greater quantity it should be distilled.

3. *Approximately 40% solution of sodium acetate.* Sodium acetate is particularly easy to recrystallize and can readily be obtained free from iron. Prepare a 40% solution of $CH_3COONa, 3H_2O$ with distilled water.

4. *Approximately 25% solution of glucose.* Most samples of glucose are quite free from iron. Prepare a 25% solution in distilled water.

5. *Dipyridyl reagent.* Dissolve 0·468 g. of dipyridyl in 6 ml. of N hydrochloric acid and make up to 100 ml. with distilled water.

6. *Standard iron solution.* Dissolve 0·350 g. of ferrous ammonium sulphate in distilled water containing a few drops of sulphuric acid and make up to 100 ml. in a standard flask. The solution contains 50 mg. of iron per 100 ml.

B.D.H.'s A.R. reagents were used throughout. With glass-distilled water and the above reagents, iron in the blanks should be negligible.

METHOD.

Measure exactly 0·1 ml. of blood into a pyrex tube which has a mark at the 10 ml. level. The pipette should be washed carefully with the minimum of water. Add 0·2 ml. of 50% sulphuric acid and about 10 drops of concentrated nitric acid. Heat carefully, with constant agitation, over a micro-burner until charring begins. Cool and add more nitric acid, repeating these operations until no further charring occurs. Boil off the excess of nitric acid. The residual drop of sulphuric acid usually contains a white precipitate which is probably anhydrous ferric sulphate. Add about 0·5 ml. of water and warm to dissolve this precipitate. Add 0·3 ml. of dipyridyl reagent, followed by 1 ml. of glucose solution. Mix well by shaking and add 5 ml. of sodium acetate solution. Heat in a boiling water bath for 5 min. for full colour development. Cool and make up to the 10 ml. mark with distilled water.

The standard is prepared in exactly the same way except that 0·1 ml. of the standard iron solution (or 1 ml. of a standard diluted ten times) is used in place of the blood.

Compare in a colorimeter with the standard preferably at 25 mm. If U is the reading for the unknown and S the reading for the standard, then $\frac{S \times 50}{U}$ = mg. of iron per 100 ml. of blood.

DISCUSSION.

Apparatus. Small colorimeter cups are now almost universally used and if these are available the entire analysis should be carried out in pyrex test-tubes 150 mm. × 10 to 12 mm. diameter. These should be accurately marked at 10 ml. If more fluid is required for the colorimeter cups slightly larger tubes should be used and these should be marked at 12 or 15 ml. Tubes should be of as small diameter as convenient in order to obtain as great a length as possible and also to ensure that the minimum amount of sulphuric acid is required to cover the bottom after incineration is complete. Pipettes should be of the wash-out type and should be accurately calibrated with mercury.

Incineration. Wet ashing is much more convenient than dry ashing. The latter takes a longer time and requires more elaborate apparatus; there is danger of loss of iron by volatilization and the iron and phosphates often tend to be in a more refractory form after ashing. With wet ashing all these difficulties are avoided but some care is necessary to prevent loss by sputtering. With ordinary care, however, this does not occur. Nitric acid was preferred to other oxidizing agents because it can easily be distilled and obtained free from iron [*cf.* Fowweather, 1934]. Excess of the reagent can be removed by boiling.

Buffering action of sodium acetate. The buffer used is ample to maintain the final *p*H well within the limits 5·0–5·5, even when two or three times the stated quantity of sulphuric acid is used.

Reduction of iron by glucose. Glucose was selected as the best reducing agent after many others had been tried. Sodium sulphite and sodium hydrosulphite are difficult to purify and are unstable in solution. Sulphur dioxide is readily available and is not subject to contamination with iron. All three of these materials, however, make the adjustment of *p*H difficult. Glucose on the other hand is easily obtained in a state free from iron and strong solutions keep well. It also has the advantage of not affecting the *p*H. Although glucose reduces ferric iron only slowly at 100° and *p*H 5·0, the presence of dipyridyl hastens the reaction so that it is completed in a very short time.

Colour production. Not less than 0·3 ml. of dipyridyl should be used. This is sufficient to promote colour formation under the experimental conditions, provided that the iron content of the blood does not exceed 75 mg. per 100 ml.—a value which is rarely encountered in practice. The colour attains its maximum intensity very quickly at the temperature of the water-bath and suffers no change if maintained at this temperature for an hour, or if kept at room temperature for as long as 24 hours. The addition of copper salts or of phosphates to the blood to be analysed in no way affects the values obtained for the iron content when estimated by this method.

The colour is not difficult to match using a daylight screen but the definition can be markedly improved by including a bluish-green light filter (which can be bought from any glazier for a few pence) in the lighting system.

Quantity of blood used. Normally, human blood contains approximately 50 mg. of iron per 100 ml., and 0·1 ml. is ample for the estimation. If the blood is markedly anaemic twice this volume should be taken. The method can also be adapted for larger quantities of blood if these are available.

RESULTS.

Some hundreds of analyses have been carried out and the reliability of the method has been checked in several ways, always with good results. Recovery of added iron from blood was excellent and some typical results are given below.

Estimated Fe content of blood mg. per 100 ml.	Estimated Fe content of blood after addition of 25 mg. Fe per 100 ml. mg. per 100 ml.	Difference
37·6	62·5	24·9
39·6	64·8	25·2
53·9	78·3	24·4
49·2	74·8	25·6
50·0	74·7	24·7

SUMMARY.

A micro-method for determining the total iron content of 0·1 ml. of blood, using dipyridyl, is described.

The author wishes to express his thanks to Dr C. H. Catlin and Mr D. T. Lewis for help with the analytical work.

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CCXXVI. HEXOSEPHOSPHATE METABOLISM OF TUMOUR EXTRACTS

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The investigation of the glycolytic mechanism of tumour tissue would be simplified if it were possible to obtain cell-free tissue extracts with properties of glycolysis analogous to those of the muscle extracts first described by Meyerhof. Barr *et al.* [1928], Warburg [1930], Boyland & Mawson [1934] and others all found, however, that injury to malignant tissue was accompanied by reduction of its power of glycolysis and that cell-free extracts were practically inactive. The substrate used in these experiments has generally been glucose, but equal lack of success has attended most attempts to obtain lactic acid from hexosephosphates by the use of tumour slices or of preparations subjected to various degrees of cell destruction. Downes [1929] observed that rat tumours would produce lactic acid from hexosephosphate at about half the rate at which they produced it from glucose, but Boyland & Mawson [1934], using hexosemonophosphate and Jensen rat sarcoma slices, obtained lactic acid at only about 1/6 the rate of formation from glucose. Results of this kind have led to a belief that the series of events in tumour glycolysis is entirely different from that observed in muscle, and doubt exists that phosphorus compounds play any part in tumour metabolism.

Boyland & Boyland [1935, 1] obtained extracts from mouse sarcomas which, after dialysis for one hour, would produce small amounts of lactic acid from hexosediphosphate, but on addition of adenylpyrophosphate the yield was greatly increased until it was about 1/4 of the amount produced by tissue slices in glucose. They suggested that previous failures to demonstrate glycolytic activity in tumour extracts might be due to rapid destruction of the coenzyme by the powerful nucleosidase known to occur in tumour tissue [Boyland & Boyland, 1935, 2]. It is noteworthy that none of the extracts used by these authors would produce lactic acid from glucose.

Scharles *et al.* [1935, 1, 2] have also described an extract of mouse sarcoma which would produce lactic acid from hexose-diphosphate and -monophosphate, but not from glycogen or glucose, but it differed from that of Boyland & Boyland in the fact that its activity did not depend on the presence of adenylpyrophosphate. The maximum rate of production of lactic acid occurred at a temperature of 50–55° and its activity was not affected by dialysis at 10° for 36 hours. The present paper is concerned with an investigation of the properties of extracts similar to those described by Scharles *et al.*

EXPERIMENTAL.

Transplantable mouse tumours have been used which have been recorded in the tables as follows:

C = carcinoma C 63. E = Ehrlich carcinoma. L = sarcoma L.M. 154. S = Vienna sarcoma.

The sarcoma L.M. 154 was originally produced by Barry & Cook [1934] by injection of 5:6-cyclopenteno-1:2-benzanthracene.

Extracts and suspensions have been made with non-necrotic material in sterile Ringer solution, and incubations have been carried out in presence of $M/30$ phosphate, the pH after additions being 7·0–7·5. Incubations were done in presence of toluene in stoppered $3\frac{1}{2} \times \frac{5}{8}$ in. tubes, and protein was precipitated with trichloroacetic acid.

Lactic acid was estimated by the method of Lohmann [1928] and alkaline precipitation of carbohydrate was avoided [Boyland & Boyland, 1935, 1]. Solutions were always distilled for 10 min. before the condensers were fitted in order to drive off volatile products. In order to prevent errors due to the fading which results from the presence of carbohydrate, the end-point used was persistence of blue colour for 30 sec. instead of 60 sec., and excess of $N/10$ iodine was added during the preliminary titration and removed with a drop of bisulphite. Yields of lactic acid have been expressed throughout as mg. lactic acid produced in one hour by an amount of extract or suspension derived from 1 g. of tissue.

Lactic acid from tumour cell suspensions.

Preliminary experiments with minced tumour tissue and with suspensions of tumour cells made by grinding the minced tissue with Ringer solution and filtering through a fine wire mesh, showed that lactic acid was produced from hexosediphosphate in considerable amount at an optimum temperature of 50–55°. A series of experiments was then carried out with cell suspensions to see whether the addition of glutathione would influence the yield of lactic acid at 38° or 52°, using glucose and hexosediphosphate as substrates. The results are given in Table I. (It should be noted that the mean values appearing in all the tables are given only for convenience in comparison of results and are not necessarily significant in themselves.) Very little lactic acid arises from glucose at 38° or 52°, and addition of glutathione does not increase this yield. The small yield of lactic acid from hexosediphosphate at 38° is not increased by addition of glutathione, but at 52° there is a considerably greater yield of lactic acid which is definitely increased by addition of glutathione. The variation in activity of the cell suspensions made the results statistically unsatisfactory, so the work was repeated using cell-free extracts and hexosediphosphate. It was not considered necessary to repeat the experiments using glucose as substrate.

Table I. *Influence of glutathione on lactic acid production of tumour cell suspensions.*

1·0 ml. suspension, 1·2 ml. additions, 6·0 mg. glucose or hexosediphosphate, 1 mg. glutathione. Incubated 1 hr.

mg. lactic acid per g. tissue per hr.

	38°		52°		38°		52°	
Tumour	Glucose	Glucose + GSH	Glucose	Glucose + GSH	Hdp	Hdp + GSH	Hdp	Hdp + GSH
E	0·00	0·01	0·00	0·19	0·42	0·75	1·94	2·43
S*	0·02	0·00	0·00	0·00	0·30	0·06	0·27	2·07
S	0·11	0·00	0·00	0·01	0·12	0·07	0·31	0·85
S	0·13	0·04	0·00	0·00	0·15	0·13	0·60	0·71
S	0·00	0·04	0·00	0·15	0·00	0·13	0·41	0·64
Mean	0·05	0·02	0·00	0·07	0·20	0·23	0·71	1·34

* 2·5 g. tumour. 11·5 ml. Ringer. Incubated 2 hours. GSH = glutathione. Hdp = hexosediphosphate.

Lactic acid from tumour extracts.

Extracts were prepared at 20–25° by mincing 4 g. tumour, grinding into a fine brei and adding 10 ml. of Ringer solution. The mixture was very thoroughly ground and centrifuged twice for 10 min. at 3500 r.p.m. Such extracts usually contained about 0·07 mg. glutathione per ml. Table II shows that at 38° addition of glutathione caused an apparent decrease in the yield of lactic acid, but the amounts determined were too small for confidence to be placed in the significance of this observation. At 52°, however, the amount of lactic acid produced after addition of glutathione was always greater than with hexosediphosphate and extract alone. These results suggested that glutathione was acting as coenzyme in the reaction, and the hypothesis was adopted that a glyoxalase was involved in the system.

Table II. *Influence of glutathione on lactic acid production of tumour extracts.*

1·0 ml. extract. 1·2 ml. additions. 6·0 mg. hexosediphosphate. 1·0 mg. glutathione. Incubated 1 hour.

mg. lactic acid per g. tissue per hr.

	38°		52°	
Tumour	Hexosedi-phosphate	Hexosedi-phosphate + GSH	Hexosedi-phosphate	Hexosedi-phosphate + GSH
S	0·36	0·11	0·78	0·99
E	0·24	0·03	1·03	1·51
E	0·61	0·50	1·52	2·36
E	0·00	0·00	0·88	1·78
E	0·27	0·00	0·55	1·17
Mean	0·26	0·13	0·95	1·56

Effects of "antiglyoxalase".

Woodward *et al.* [1935] have shown that rat kidney contains a substance which can act as a powerful antiglyoxalase, and experiments were therefore carried out to determine whether such a preparation would prevent the formation of lactic acid from hexosediphosphate at 52°. Mouse kidneys were cut into small pieces and dropped into 20 volumes of acetone, which was replaced after 10 min. by fresh acetone. After a further 10 min. the kidney was washed with ether and left to dry in the refrigerator overnight. It was then ground into a fine powder. Extracts of this material were made by grinding 100 mg. of powder with 1·0 ml. water and centrifuging twice. It was found (Table III) that addition of this antiglyoxalase preparation caused a considerable decrease in the amount of lactic acid produced from hexosediphosphate and tumour extract at 52°. The yield obtained was, in fact, about what would have been expected at 38° in absence of antiglyoxalase (Table II).

Table III. *Influence of kidney antiglyoxalase on lactic acid production of tumour extracts.*

1·0 ml. extract. 1·2 ml. additions. 6·0 mg. hexosediphosphate. 0·2 ml. antiglyoxalase. Incubated 1 hr. at 52°.

mg. lactic acid per g. tissue per hr.

Tumour	Hexosedi-phosphate	Hexosediphosphate + antiglyoxalase
C	1·03	0·16
E	1·07	0·40
L	1·60	0·49
C	0·36	0·00
C	0·98	0·43
E	1·12	0·23
Mean	1·03	0·29

Dialysed extracts.

Scharles *et al.* [1935, 1] stated that the glycolytic activity of their extracts was not reduced by dialysis in cellophane sacs for 36 hours at 10°. The results just described, however, pointed so definitely to the significance of glutathione that a series of dialysis experiments was carried out. Thin sacs were made of B.D.H. collodion "Special for preparing permeable membranes" in 4 × ½ in. tubes. Dialysis was carried out in Ringer solution at 7°, 1·2 litres of Ringer being used for each ml. of extract. During dialysis a parallel sample of extract was kept at 7° in a glass tube and in each experiment dialysed and undialysed extracts were investigated simultaneously.

Contrary to the observation of Scharles *et al.* it was found (Table IV) that the glycolytic activity of the extract was very much reduced by dialysis. The undialysed extract, kept at the same temperature for the same length of time, retained much of its original glycolytic power. In one experiment, for example, the fresh extract produced 1·60 mg. lactic acid per g. tissue per hour and after keeping for 24 hr. at 7° the yield was 1·26 mg./g./hr. in spite of the fact that the extract had been diluted 14 % to compensate for the liquid taken up by the dialysed extract. The latter only produced 0·43 mg./g./hr. The addition of glutathione to dialysed extracts always caused a large increase in their lactic acid production, and although this did not always reach the level attained by the undialysed extract plus glutathione there seems to be little doubt that the loss of glycolytic activity observed after dialysis is due to the disappearance of glutathione.

Table IV. *Influence of dialysis and addition of glutathione on lactic acid production of tumour extracts.*

1·0 ml. extract. 1·2 ml. additions. 6·0 mg. hexosediphosphate. Incubated 1 hr. at 52°.

mg. lactic acid per g. tissue per hr.

	Undialysed		Dialysed		
Tumour	Without GSH	With GSH	Without GSH	With GSH	Time of dialysis hr.
E	0·37	0·75 (1·3)*	0·09	0·65 (1·3)*	26
L	0·82	1·30 (1·5)	0·05	0·70 (1·5)	20
L	0·73	1·17 (2·0)	0·01	1·22 (2·0)	68
L	1·26	1·44 (3·0)	0·43	1·66 (3·0)	24
E	1·12	1·42 (3·0)	0·11	1·08 (3·0)	48
Mean	0·86	1·22	0·14	1·06	

* Figures in brackets represent mg. glutathione added.

DISCUSSION.

The normal source of lactic acid in the tumour cell is probably glucose. Tumour slices will produce lactic acid from glucose at a very high rate, but damage to the tissue cells destroys their glycolytic power to such an extent that suspensions of cells are quite inactive. If the loss of activity were strictly proportional to the degree of breakdown of cell structure this result would hardly be anticipated, for the suspensions used in this work (Table I) contained about 10^6 apparently undamaged cells per ml., and though they often gave no lactic acid at all with glucose they were capable of producing tumours. Boyland & Boyland [1935, 1] were able to obtain lactic acid from hexosediphosphate and tumour extracts on addition of adenylpyrophosphate, but no lactic acid could be obtained with glucose. If we assume that hexosediphosphate is a normal metabolic product of tumour, destruction of cells must damage the enzyme system at some point before the production of hexosediphosphate as well as causing decomposition of adenylpyrophosphate, and in tumour suspensions this damage extends to cells which remain histologically intact. It has, however, been shown that adenylpyrophosphate is not necessary for the conversion of hexosediphosphate into lactic acid by tumour extracts, but for this reaction to take place in the absence of the muscle coenzyme it is necessary for the temperature to be comparatively high and glutathione must be present. Even under these conditions no lactic acid is produced from glucose.

Meyerhof [1933] has dismissed methylglyoxal as of no importance in muscle glycolysis. It has no place in the Embden-Meyerhof scheme and Lohmann [1932] has shown that lactic acid production by muscle extract can take place in absence of glutathione, the coenzyme of glyoxalase. However Gaddie & Stewart [1935] have reopened this question with the observation that addition of glutathione to dialysed muscle extract containing magnesium ions does lead to considerable lactic acid production from glycogen, and add the interesting observation that glutathione appears to stimulate the production of methylglyoxal as well as to aid in its conversion into lactic acid. It seems, in fact, that production of methylglyoxal from glycogen or glucose may be conditioned by the possibility of its rapid disappearance, for Toenniessen & Fischer [1926] found that methylglyoxal was produced from hexosediphosphate, but not from glucose or glycogen, in presence of muscle brei and pancreatic antiglyoxalase. In the case of tumour tissue no analogy can be found at 38° with the observations of Gaddie & Stewart, but at 52°, using hexosediphosphate as substrate, the resemblance is very close. It is known [Lohmann, 1932] that glyoxalase is very stable to heat and it may be that some change of a non-enzymic nature takes place at 52° as a stage between hexosediphosphate and methylglyoxal which is a necessary step in tumour metabolism but which can be achieved by an enzymic process at 38° with muscle extracts. It is difficult to imagine an enzyme of mammalian tissue which would be active at 52° and inactive at 38°.

The observation of Scharles *et al.* [1935, 1] that no methylglyoxal is detectable among the end-products of the action of tumour extracts on hexosediphosphate at 52° is doubtless due to the fact that it is decomposed as soon as it is formed, but it is more difficult to explain their failure to observe any decrease of activity in their extracts after dialysis. In the present work every dialysed extract has shown greatly decreased glycolytic power, which has invariably been increased on addition of glutathione. This observation, together with the fact that kidney antiglyoxalase reduces the production of lactic acid from hexosediphosphate at 52° to about the level observed at 38° in absence of added glutathione, leaves no doubt that the extra glycolysis observed at the higher temperature is due to a system, involving glutathione as coenzyme, which is probably a glyoxalase.

In a comparison of the glycolytic properties of muscle and tumour extracts the following points are of importance:

(1) Muscle extract will form lactic acid from carbohydrate or hexosephosphate in presence of adenylpyrophosphate at 38°, but tumour extract will only utilize hexosephosphate for this purpose.

(2) Muscle extract will form lactic acid from carbohydrate in the presence of glutathione and magnesium at 38°, but tumour will not utilize either carbohydrate or hexosediphosphate at this temperature in the absence of adenylpyrophosphate.

(3) Tumour extract will form lactic acid from hexosediphosphate in the absence of adenylpyrophosphate at 52°, but only in the presence of glutathione.

Two distinct differences can now be detected in the glycolytic system of tumour extract as compared with that of muscle. Firstly, hexosediphosphate cannot be produced from carbohydrate. If it could, lactic acid could be produced from glucose at 38° on addition of adenylpyrophosphate and also at 52° on addition of glutathione. Secondly, lactic acid cannot be produced by tumour extract at 38° from hexosediphosphate even on addition of glutathione. The latter defect can be remedied by addition of adenylpyrophosphate It is unlikely that the destruction of this compound by nucleosidase is the whole reason for the difference because presumably muscle extract can form lactic acid from hexosediphosphate as well as from glycogen on addition of glutathione and magnesium. An enzyme capable of carrying out the reaction is certainly present in tumour extract because lactic acid is produced from hexosephosphate at 52° if glutathione is present, and the link in the chain which is absent seems to be the capacity to convert hexosediphosphate into methylglyoxal at 38°.

SUMMARY.

1. Lactic acid is not produced from glucose by mouse tumour cell suspensions at 38° or 52°, with or without addition of glutathione.

2. Only small amounts of lactic acid are produced by tumour cell suspensions or extracts from hexosediphosphate at 38°, and these are not increased by addition of glutathione.

3. At 52° tumour cell suspensions and extracts produce considerable amounts of lactic acid from hexosediphosphate, and these yields are greatly increased by addition of glutathione.

4. Dialysed tumour extracts produce very little lactic acid from hexosediphosphate at 52°, but their glycolytic activity is restored by addition of glutathione.

5. The lactic acid production of tumour extracts at 52°, with hexosediphosphate as substrate, is greatly reduced by addition of kidney antiglyoxalase.

6. The contrast between the glycolytic systems of extracts of muscle and tumour is discussed.

Grateful acknowledgment is made to Prof. E. L. Kennaway, for the gift of mice bearing the sarcoma L.M. 154.

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CCXXVII. SYNTHESIS OF PEPTIDES CONTAINING CYSTINE AND GLUTAMINE, WITH REMARKS ON THEIR POSSIBLE BEARING ON THE STRUCTURE OF INSULIN AND A NOTE ON THE AMIDE NITROGEN OF INSULIN.

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Whilst it is universally accepted that insulin, as isolated from the pancreas, is a protein, the possibility is not yet excluded that this protein may owe its physiological activity to a specific grouping. The suggestion was made by Harington & Scott [1929] that an analogy might exist between insulin and thyroglobulin which is physiologically active by virtue of its content of thyroxine, although it was recognized by these authors that the separation of the active constituent from the whole protein which can be effected in the case of thyroglobulin might prove quite impracticable in that of insulin.

All attempts which have been made to separate an active substance of low molecular weight from insulin have in fact proved fruitless, since the mildest degradation of the molecule, by proteolytic enzymes or by other methods, has invariably resulted in total loss of physiological activity. In spite of these failures, however, the impression that insulin does indeed possess an active "prosthetic" group has persisted and the evidence bearing on such an assumption has been summarized by Jensen & Evans [1934].

From the numerous experiments which have been made on the physiological inactivation of insulin by treatment with mild hydrolytic agents two main conclusions have emerged: (1) a significant proportion of the nitrogen of insulin is liberated as ammonia under conditions which suggest that it occurs in the protein as a labile amide and such loss of ammonia involves loss of activity; (2) the sulphur of insulin is very labile towards alkali and treatment with alkali sufficient to cause elimination of even a part of the sulphur also inactivates insulin. Lability of amide nitrogen in proteins is generally associated with the occurrence therein of glutamine and the sulphur of cystine is well known to become highly labile when the amino-acid occurs in certain types of peptide linkage. It is known from other sources that insulin yields on hydrolysis a large proportion both of glutamic acid and cystine, and Jensen & Evans [1934] were thus led to suggest that the hypothetical active group of insulin might contain cystine and glutamine in peptide linkage with one another.

Until quite recently it has been impossible to prepare peptides containing cystine and glutamine; within the last two or three years, however, methods have become available [Bergmann & Zervas, 1932; Bergmann *et al.*, 1933; du Vigneaud *et al.*, 1930; Sifferd & du Vigneaud, 1935; Harington & Mead, 1935] which offered some prospect of success and it was therefore considered worth while to attempt the synthesis of cysteylglutamine and glutaminylcysteine in order to subject the suggestion of Jensen & Evans to direct test.

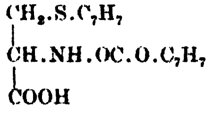
Whilst it was recognized that the possibility that either of these simple compounds would itself exhibit hypoglycaemic activity was remote it was hoped that a study of their respective chemical behaviours in relation to that of insulin might afford definite evidence in favour of or against the occurrence of a similar grouping in the hormone.

Synthetical experiments.

The syntheses, which follow accepted lines and are briefly described below, call for no detailed comment. Two general points in connection with them may however be mentioned. The first concerns the preparation of cystine derivatives and the second the use of benzyl esters in peptide synthesis. It was found while this work was in progress that even the simpler derivatives of cystine are very difficult to crystallize. Two crystalline compounds of this amino-acid are described in the appendix to the experimental section but these were but two successes among many failures. When the syntheses of cystylglutamine and glutaminylcystine were first undertaken, the route chosen passed through a series of cystine compounds and although both peptides were eventually obtained as crystals in their reduced forms, no success attended many attempts to crystallize several of the intermediates. When, however, cysteine derivatives were substituted for those of cystine no difficulties were encountered. It was necessary at times to protect the sulphydryl group and here the use of *S*-benzyl derivatives, which has been introduced by du Vigneaud, was found to be very satisfactory.

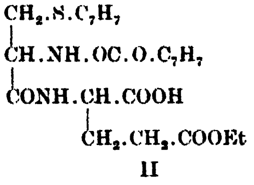
It will be recalled that the method of peptide synthesis devised by Bergmann & Zervas [1932] depends upon the removal of *N*-benzylcarbonyl- and of *O*-benzyl-groups by means of a reduction process. It was observed during the present work that removal of a benzyl ester group by reduction with phosphonium iodide proceeds very much more slowly than the removal of an *N*-benzylcarbonyl-group under identical conditions. One experiment with an *S*-benzyl-compound indicated that removal of this group probably proceeds more slowly still. These differences in ease of reduction may be of use in synthetic work. An example occurs in the preparation of cysteine benzyl ester hydroiodide (p 1605).

Cysteylglutamine. *N*-Benzylcarbonyl-*S*-benzylcysteine (I), prepared in the usual manner from *S*-benzylcysteine and benzylcarbonyl chloride, was converted into the acid chloride and coupled in aqueous solution in presence of triethylamine with γ-monoethyl glutamate to give γ-monoethyl-*N*-benzylcarbonyl-*S*-benzylcysteylglutamate (II): the latter was treated with ammonia and the resulting amide was freed from benzyl and benzylcarbonyl residues by reduction with sodium in liquid ammonia to yield cysteylglutamine (III).



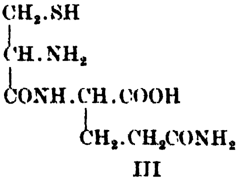
$$\begin{array}{l} CH_2.S.C_7H_7 \\ | \\ CH.NH.OC.O.C_7H_7 \\ | \\ COOH \end{array}$$

I



$$\begin{array}{l} CH_2.S.C_7H_7 \\ | \\ CH.NH.OC.O.C_7H_7 \\ | \\ CONH.CH.COOH \\ \qquad\quad | \\ \qquad\quad CH_2.CH_2.COOEt \end{array}$$

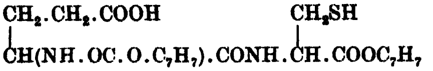
II



$$\begin{array}{l} CH_2.SH \\ | \\ CH.NH_2 \\ | \\ CONH.CH.COOH \\ \qquad\quad | \\ \qquad\quad CH_2.CH_2CONH_2 \end{array}$$

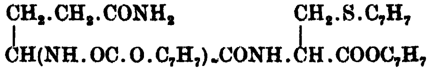
III

Glutaminylcysteine. *N*-Benzylcarbonylcystine benzyl ester, prepared by the action of *N*-benzylcarbonylcystyl chloride on benzyl alcohol was converted into cysteine benzyl ester hydroiodide by partial reduction with phosphonium iodide: the cysteine benzyl ester was then coupled with *N*-benzylcarbonylglutamic anhydride to yield *N*-benzylcarbonyl-α-glutamylcysteine benzyl ester (IV); the *S*-benzyl derivative of the latter was converted in turn into the acid chloride and the amide (V) which was finally reduced with sodium in liquid ammonia to give glutaminylcysteine (VI).



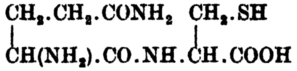
$$\overset{CH_2.CH_2.COOH}{\overset{|}{C}}H(NH.OC.O.C_7H_7).CONH.\overset{CH_2SH}{\overset{|}{C}}H.COOC_7H_7$$

IV



$$\overset{CH_2.CH_2.CONH_2}{\overset{|}{C}}H(NH.OC.O.C_7H_7).CONH.\overset{CH_2.S.C_7H_7}{\overset{|}{C}}H.COOC_7H_7$$

V



$$\overset{CH_2.CH_2.CONH_2}{\overset{|}{C}}H(NH_2).CO.NH.\overset{CH_2.SH}{\overset{|}{C}}H.COOH$$

VI

Physiological tests.

We are indebted to Dr H. P. Himsworth for testing the two synthetic peptides for hypoglycaemic activity. The peptides were tested as such and after oxidation to the disulphide forms by means of hydrogen peroxide. In each case the material under test (10 mg.) was dissolved in sterile saline (2·5 ml.) and injected into a rabbit, the blood sugar being determined at intervals for a period of 1 hour. No suggestion of hypoglycaemic activity was obtained. It may be noted that glycylcystine, cystylglycine and γ-glutamylcystine have recently been found to be devoid of physiological activity [Jensen *et al.*, 1936].

Labile sulphur.

Table I gives the proportions of labile sulphur in insulin, glutathione and the new synthetic peptides together with some values from the literature. The substances were boiled in 0·1 *N* sodium carbonate solution during 45 min. and the figures are percentages of the total sulphur eliminated as hydrogen sulphide upon acidification and boiling after the treatment with carbonate. The conditions were standardized as far as possible but appear to be more severe than those used by earlier workers since our result for insulin is considerably higher than that previously reported.

Table I.

Substance	"Sodium carbonate-labile sulphur" (%)
Glutaminylcystine	8·2
Cystylglutamine	17·8
Glutathione (oxidized)	37·3
Insulin (crystalline)	60·0
Cystine*	2·8
Alanylcystine*	18·6
Alanylcystine anhydride*	91·8
Insulin†	37·4

* Brand & Sandberg [1926]. † Abel *et al.* [1927].

It will be seen that cystylglutamine and glutaminylcystine resemble alanylcystine and are intermediate in their behaviour between cystine itself and the more complex glutathione. Insulin is much less stable.

Liberation of ammonia during treatment with acid and alkali.

The peptides were treated with 0·1 *N* HCl and with *N*/30 NaOH under the conditions prescribed for insulin by Jensen & Evans [1932]. The values for insulin are taken from these authors. The results are presented as percentages of the total amide nitrogen.

The liberation of ammonia from insulin in acid was measured under conditions in which the substance would be present as the insoluble "heat precipitate".

Table II. *Percentage of nitrogen liberated as ammonia by treatment with acid and alkali.*

	Treatment	
Substance	$N/10$ HCl at 100° 1 hour	$N/30$ NaOH at 34° 3 hours
Glutaminylcystine	100	9·4
Cystylglutamine	69·5	5·7
Insulin [Jensen & Evans, 1932]	(23)	10·9

A comparison therefore of the result so obtained with those from the (soluble) peptides would have little significance.

Whereas the sulphur of cystylglutamine is more labile than that of glutaminylcystine in the case of the amide nitrogen the position is reversed. If the doubtful result obtained by acid treatment of insulin is neglected it appears that the lability of the amide nitrogen in insulin is little different from that in the peptides; the sulphur of insulin on the other hand is far more labile than that of either of the peptides. In so far as deductions can be made from the study of the simple peptides the properties of the latter therefore afford no support to the suggestion that the cystylglutamine or glutaminylcystine linkage occurs in the molecule of insulin. This conclusion must be accepted with reserve however since the lability of the sulphur in such peptides may well be increased by attachment of additional amino-acid residues.

Amide nitrogen and glutamic acid content of insulin.

Glutamic acid has been isolated from insulin by Jensen & Wintersteiner [1932]. Since these workers were unable to detect the presence of aspartic acid, it may be assumed provisionally that glutamic acid is the only dicarboxylic acid present. It may also be assumed that this acid is in combination with the peptide structure of the molecule through its α-amino- and α-carboxyl-groups only, the γ-carboxyl-group either remaining "free" or in combination as an (acid) amide. As the total number of free acidic groups in the insulin molecule is now known with some accuracy [Harington & Neuberger, 1936] it follows that an estimate of the amount of glutamic acid present may be made if the amide nitrogen content is also known. It should be pointed out that this estimate will be subject to two sources of error. First it is not certain that all free acidic groups are revealed by titration and secondly a small part of the base binding is attributable to terminal carboxyl groups. These errors will affect the result in opposite directions.

Our present knowledge of the amide nitrogen content of insulin is based on the amount of ammonia liberated during complete hydrolysis [Wintersteiner *et al.*, 1928]. It has been shown by Shore *et al.* [1935] however that, in the case of some proteins at least, ammonia is liberated during hydrolysis from sources other than amide groups. It therefore appeared desirable to determine the amide nitrogen of insulin by the method described by these workers.

Experiments on the hydrolysis of insulin by 0·2 N HCl at 100° were unsatisfactory because the substance remained undissolved even after 20 hours. Determinations were therefore made of the ammonia liberated by the treatment of insulin with 5N HCl for varying periods of time. The results which are given in Fig. 1 lead to a value of approximately 34 equivalents of amide nitrogen per molecule assuming the molecular weight of insulin to be 35,100. The quantities of insulin employed and therefore the accuracy of the results were severely limited by the expensive nature of the material. It is hoped however that sufficient accuracy has been achieved to make possible a rough estimate of the amount of glutamic acid in insulin. If the number of free acidic groups due to glutamic acid is assumed to be roughly 32, it follows that the proportion of this acid in insulin is about 30 %. This figure is of course subject to the limitations mentioned above.

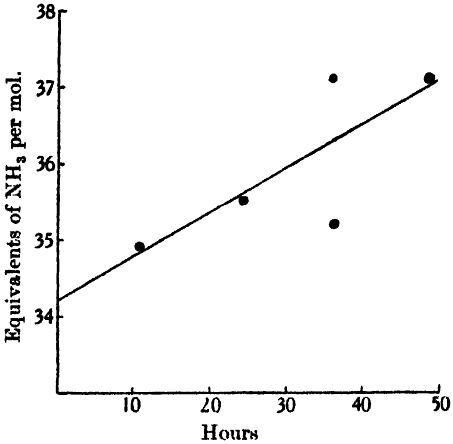


Fig. 1. Liberation of ammonia from insulin during treatment with $5N$ HCl at 100°.

EXPERIMENTAL.

Synthesis of cysteylglutamine.

S-*Benzylcysteine.* Cystine was reduced by means of zinc dust and HCl to cysteine which was isolated as its cuprous mercaptide. The latter was decomposed with H_2S and the solution after removal of cuprous sulphide was concentrated, made alkaline and treated with benzyl bromide in the usual manner. The product was recrystallized from water and formed large elongated plates or prisms which melted at 219° with decomposition after sintering at 205°. The yield was 58 % of the theoretical.

It is probable that this compound may be prepared more conveniently by the method of du Vigneaud *et al.* [1930].

N-*Benzylcarbonyl*-S-*benzylcysteine.* *S*-Benzylcysteine (5·0 g.) in $2N$ NaOH (12 ml.) was treated with benzylcarbonyl chloride (5 ml.; 25 % excess) and $2N$ NaOH (15 ml.) with vigorous shaking and ice cooling during 30 min. The mixture was finally shaken mechanically for a further 30 min. During the course of the reaction the sodium salt of the product separated as a heavy layer. The whole was washed three times with ether. The latter was discarded and the two heavier layers were made acid to Congo red by the addition of $5N$ HCl and were extracted three times with ethyl acetate. The ethyl acetate solution was washed well with saturated NaCl, dried over sodium sulphate, concentrated to a small volume under diminished pressure and treated cautiously with ligroin. Crystallization started at once and was completed by the addition of ligroin and by standing at 0°. The substance was collected and recrystallized from benzene. The yield was 90 % of the theoretical. *N*-Benzylcarbonyl-*S*-benzylcysteine forms colourless elongated prisms melting at 93–95° after sintering. (Found: N, 4·17; S, 9·10 %. $C_{18}H_{19}O_4NS$ requires N, 4·06; S, 9·28 %. 42·5 mg. required 6·10 ml. $N/50$ NaOH for neutralization to cresol red. $C_{18}H_{19}O_4NS$ titrating as a monobasic acid requires 6·16 ml.)

N-Benzylcarbonyl-*S*-benzylcysteine was also prepared as follows: *N*-benzylcarbonylcystine in 80 % alcoholic sulphuric acid (0·75 *N*) was reduced with zinc-copper couple to *N*-benzylcarbonylcysteine which formed prismatic needles (M.P. 82°) from ethyl acetate-ligroin. *N*-Benzylcarbonylcysteine was treated with benzyl bromide in alkaline solution giving *N*-benzylcarbonyl-*S*-benzylcysteine in 63 % yield.

*γ-Monoethyl-*N*-benzylcarbonyl-*S*-benzylcysteylglutamate.* *N*-Benzylcarbonyl-*S*-benzylcysteine (4·0 g.) was suspended in dry ether (30 ml.). The mixture was cooled in ice-salt and shaken with phosphorus pentachloride (2·8 g.: 15 % excess). The undissolved material soon passed into solution and after about 5 min. nearly all the phosphorus pentachloride had disappeared. The pale yellow solution was decanted from excess reagent and evaporated to a syrup at a low temperature and with exclusion of atmospheric moisture. The residue was treated with light petroleum (B.P. 60–80°) and soon crystallized in small prisms. The acid chloride was collected, washed with light petroleum and added slowly to a mechanically stirred ice-cold solution of γ-ethylglutamate (3·0 g.; 50 % excess) and triethylamine (4·0 ml.) in water (15 ml.). When rapid stirring had been maintained for 10 min. triethylamine (1·0 ml.) and a small quantity of ether were added and any lumps were broken up with a rod. After being stirred during a further 10 min. the mixture was centrifuged. The upper layer was discarded and the aqueous layer was decanted from a small residue, acidified and extracted three times with ethyl acetate. The ethyl acetate solution was washed with dilute HCl and several times with saturated NaCl. It was dried over sodium sulphate in the presence of norite, concentrated under diminished pressure to a small volume and treated cautiously with ligroin The oil which separated crystallized after a short period at 0°. The material after collection weighed 3·6 g. or 62 % of the theoretical amount. It was recrystallized from ethyl acetate and formed fine needles which melted at 88° after sintering at 85°. (Found: N, 5·47; S, 6·42 %. $C_{25}H_{31}O_7N_2S$ requires N, 5·58; S, 6·37 %). 49·7 mg. in methyl alcohol required 5·01 *N*/50 NaOH for neutralization to cresol red. $C_{25}H_{31}O_7N_2S$ titrating as a monobasic acid requires 4·95 ml.

N-*Benzylcarbonyl*-S-*benzylcysteylglutamine.* γ-Monoethyl-*N*-benzylcarbonyl-*S*-benzylcysteylglutamate (7·3 g. of recrystallized material) was dissolved in concentrated ammonia solution (140 ml. of sp. gr. 0·880). After standing in a warm place overnight the solution was centrifuged in order to remove a trace of flocculent material and was concentrated under diminished pressure until excess of ammonia had been removed. The residue was diluted to 100 ml. with water and treated with a slight excess of 2 *N* HCl. A white viscous oil separated which hardened rapidly. It was collected and crystallized from *n*-propyl alcohol. The yield was 5·3 g. or 78 % of the theoretical. After a second crystallization from *n*-propyl alcohol the substance formed fine needles which melted at 188–189° after sintering at 186°. (Found: N, 8·8: S, 6·31 %. $C_{23}H_{27}O_6N_3S$ requires N, 8·9; S, 6·76 %.)

Cysteylglutamine. *N*-Benzylcarbonyl-*S*-benzylcysteylglutamine (3·6 g.) was dissolved in liquid ammonia (35 ml.) in an apparatus fitted with a mechanical stirrer. The solution was treated gradually with sodium in small pieces (0·7 g.: 4 equiv.) and was cooled in an acetone-CO_2 mixture until nearly all the sodium had been added. When the blue colour due to excess sodium had become permanent the bulk of the ammonia was allowed to evaporate and the last traces were removed by suction. The residue was suspended in dry alcohol (15 ml.) cooled to −30° and treated with sufficient HI to give a faintly acid solution. The latter was diluted with water, extracted with ether in order to remove dibenzyl and then treated cautiously with $5N$ ammonia until the pH had risen to about 5·0. The solution was then evaporated to a small volume under diminished pressure and treated rapidly with alcohol. The granular precipitate was collected and washed with alcohol at the centrifuge until the washings no longer gave a test for iodide. The crude peptide was finally collected. After being dried over phosphoric oxide *in vacuo* it weighed 1·4 g. or 74 % of the theoretical amount. Attempts to crystallize the crude peptide were unsuccessful.

The substance in portions of 200 mg. was dissolved in water (3·0 ml.). The solution was treated with 10 drops of 0·5 N H_2SO_4 and then with a suspension of cuprous oxide first in the cold and then with gentle warmth until a small dark red deposit appeared. The solution generally began to darken at this point. The cuprous compound which was grey-violet in colour and which dissolved in water to form a clear violet solution, separated when the solution was cooled. Precipitation was completed by adding 3 vol. of alcohol. The precipitate was suspended in a small volume of water containing 3 drops of 0·5 N H_2SO_4 and was then treated with alcohol. The precipitates from all the 200 mg. portions were united at this stage and were washed 10 times at the centrifuge by suspension in water followed by treatment with alcohol. The material was finally suspended in water and decomposed with H_2S. The cuprous sulphide was removed and washed with water at the centrifuge. The solution and washings were concentrated to a small volume under diminished pressure and adjusted to pH 4·8 by the addition of $5N$ ammonia. Crystallization started at once and continued during further evaporation of the solution over phosphoric oxide *in vacuo*. The product (290 mg.) was collected, washed with water, 50 % alcohol and absolute alcohol and was dried at 56° *in vacuo*. A second crop (422 mg.) of less pure material was obtained by allowing the mother liquor to stand over alcohol in a desiccator at a pressure of 300 mm.

It may be of interest to record that when the mother liquors from the preparation of the violet-coloured cuprous mercaptide were worked up by way of the compound formed with mercuric sulphate, the peptide recovered from the latter derivative gave a white cuprous mercaptide which was not appreciably soluble in water.

Cysteylglutamine forms colourless plates or prisms, and is more soluble in water than the isomeric glutaminylcysteine. (Found: C, 38·2; H, 6·26; N, 16·6; S, 12·40; amide N, 5·5, 5·56 %. $C_8H_{15}O_4N_3S$ requires: C, 38·6; H, 6·02; N, 16·9; S, 12·85; amide N 5·6 %.)

Optical rotation. Cysteylglutamine. 15·1 mg. in 0·5 ml. water had $\alpha_{5461} + 0{\cdot}1°$ in a 0·5 dm. tube whence $[\alpha]_{5461} = +6{\cdot}6°$.

Cystylglutamine. 10·3 mg. of the peptide in 0·797 ml. of water containing the theoretical amount of hydrogen peroxide and a trace of copper sulphate had $\alpha_{5461} - 0{\cdot}09°$ in a 0·5 dm. tube when oxidation was complete. $[\alpha]_{5461} = -13{\cdot}9°$.

Iodine titration. The peptide (9·8 mg.) was dissolved in 7·5 % potassium iodide solution (5 ml.) and treated with approximately 0·01 N iodine (5·0 ml.). The excess of iodine was back-titrated with 0·01 N sodium thiosulphate. 3·81 ml. of 0·01 N iodine were required, the theoretical quantity being 3·94 ml. of 0·01 N.

Synthesis of glutaminylcysteine.

N-*Benzylcarbonylcystine benzyl ester.* N-Benzylcarbonylcystyl chloride from 20 g. of N-benzylcarbonylcystine [Harington & Mead, 1935] was made into a thick paste with ether and added to a mixture of benzyl alcohol (26 g.: 200 % excess) with 2 vol. of dry chloroform. Next day the filtered solution was evaporated under diminished pressure and the syrupy residue dissolved in ethyl acetate. The ethyl acetate solution was washed with water, saturated NaCl, sodium bicarbonate solution and finally twice with saturated NaCl. It was then dried with anhydrous sodium sulphate, concentrated to a syrup and stirred well with 5–10 vol. of ligroin. After standing for a short time in the refrigerator the lower layer had set to a firm jelly, from which the ligroin was decanted. The lower layer was ground with ligroin and collected. The substance was dried *in vacuo* over several changes of sulphuric acid. Yield 22·7 g. = 84 % of a rather sticky amorphous powder.

Cysteine benzyl ester hydroiodide. *N*-Benzylcarbonylcystine benzyl ester (13·9 g.) in glacial acetic acid (140 ml.) was treated with phosphonium iodide (22·8 g.) at 50° in the manner described by Harington & Mead [1935]. After 70 min. evolution of carbon dioxide was almost at an end. The solution was then decanted from excess reducing agent and concentrated under diminished pressure to a small volume. Crystallization of the product was started by scratching and was completed by the cautious addition of dry ether, and by keeping at 0° for 2 hours. The product was collected and washed with acetic acid and dry ether. It was recrystallized from glacial acetic acid and formed colourless plates melting with decomposition at 139° after sintering at 135°. (Found: N, 4·4 %; I, 36·6, 37·4 %. $C_{10}H_{14}O_2NIS$ requires N, 4·1; I, 37·5 %).

N-*Benzylcarbonyl-α-glutamylcysteine benzyl ester.* Cysteine benzyl ester hydroiodide (10·15 g. of recrystallized material) was suspended in dry chloroform (40 ml.). The suspension was cooled in ice and treated with diethylamine (3·0 ml.) which caused the hydroiodide to pass into solution. To the latter cooled in ice-salt was added a solution of *N*-benzylcarbonylglutamic anhydride (7·9 g.) in dry chloroform (30 ml.). The solution was kept in the freezing mixture for 30 min. and then overnight at room temperature. The partly gelatinous material which had separated was collected and the filtrate evaporated to dryness under diminished pressure. The residue together with the first crop was crystallized from boiling propyl alcohol and recrystallized from diluted ethyl alcohol. The product formed colourless elongated prisms melting at 148°. Yield 9 g. = 64 % of the theoretical. (Found: N, 5·85; S, 6·88 %; $C_{23}H_{26}O_7N_2S$ requires N, 5·9; S, 6·75 %.)

N-*Benzylcarbonyl-α-glutamyl*-S-*benzylcysteine benzyl ester.* *N*-Benzylcarbonyl-α-glutamylcysteine benzyl ester (3·0 g. of recrystallized material) was suspended in dry chloroform (7·5 ml.) and dissolved by addition of diethylamine (1·3 ml.: 2 mol.). The solution was treated with benzyl bromide (0·92 ml. : 1 mol.). The temperature rose to about 40° after the addition. After about 10 min. further quantities of diethylamine (0·26 ml.) and benzyl bromide (0·2 ml.) were added. When the mixture had stood at room temperature for a further 45 min. it was evaporated to a syrup under diminished pressure. The syrup was dissolved in alcohol and the evaporation repeated in order to remove the last traces of chloroform. The residue was dissolved in a small volume of alcohol and the solution was treated with a slight excess of HCl (2*N*) and then cautiously with water. After a short time at 0° the crystalline product was collected and washed with alcohol. It was recrystallized from *n*-propyl alcohol with the use of norite. Yield 3·0 g. or 79 % of the theoretical. The substance forms colourless needles or prisms melting at 144°. (Found: N, 4·9; S, 5·41 %. $C_{30}H_{32}O_7N_2S$ requires N, 4·96; S, 5·6 %. 45·7 mg. required 4·06 ml. *N*/50 NaOH to neutralize to cresol red; $C_{30}H_{32}O_7N_2S$ titrating as a monobasic acid requires 4·05 ml.)

N-*Benzylcarbonyl-α-glutaminyl*-S-*benzylcysteine benzyl ester.* *N*-Benzylcarbonyl-α-glutamyl-*S*-benzylcysteine benzyl ester (2·3 g. of recrystallized material) was suspended in dry ether (25 ml.). Phosphorus pentachloride (1·2 g.; 15 %

excess) was added to the suspension which was cooled intermittently in ice-salt and vigorously shaken. Separation of the acid chloride as fine needles started before the suspension had become quite clear. Shaking was continued for about 10 min. and light petroleum (50 ml. of B.P. 60–80°) was then added in order to complete the separation of the product. The latter was collected, washed with light petroleum, and suspended in dry chloroform (40 ml.). The suspension was added slowly with constant agitation to ether (100 ml.) at $-10°$ which had been previously saturated with dry ammonia at that temperature. A white granular precipitate settled out. Any lumps were broken up with a rod and the mixture was left at room temperature overnight. The precipitate was collected, washed with ether and dried at 40°. It was then ground with water, collected, washed with ether and dried. The product was recrystallized from *n*-propyl alcohol (150 ml.) with the use of norite. Yield 1·6 g. or 70 % of the theoretical. The substance forms fine needles melting at 182° after sintering. (Found: N, 7·47; S, 5·74 %. $C_{30}H_{33}O_6N_3S$ requires N, 7·46; S, 5·68 %.)

Glutaminylcysteine. Removal of the *N*-benzylcarbonyl-, *S*-benzyl- and benzyl ester-groups from the preceding compound was accomplished by reduction with sodium in liquid ammonia [Sifferd & du Vigneaud, 1935]. No tendency was observed for the benzyl ester group to be replaced under these conditions by an amide group.

N-Benzylcarbonyl-α-glutaminyl-*S*-benzylcysteine benzyl ester (5·0 g.) was suspended in liquid ammonia (*ca.* 50 ml.) in an apparatus fitted with a mechanical stirrer. The suspension was treated with sodium in small pieces (1·23 g. : 6 equiv.). The sodium was added rapidly at first and the mixture kept at $-60°$ during this period. The last portions of sodium were added more slowly while the temperature was allowed to rise to the boiling point of ammonia. A deep blue colour due to excess of sodium appeared soon after the addition of the last piece. The crude product was isolated exactly as in the case of cysteylglutamine, the total yield being 60 %.

The crude peptide was suspended in a small volume of water and treated with $2N$ H_2SO_4 until solution was almost complete. After filtration the solution was treated with an excess of Denigès mercuric sulphate reagent. The mercury compound was collected at the centrifuge and a second crop obtained by dilution of the mother liquor with water. The precipitates were united, washed with water several times, suspended in water and gassed with H_2S during several hours. The mercuric sulphide was centrifuged, resuspended in water, again treated with H_2S, and finally centrifuged. The combined aqueous liquors were evaporated to a small volume under diminished pressure, then warmed to about 35° and treated with a suspension of cuprous oxide. The latter dissolved but no precipitate appeared until several additions of the reagent had been made and the inside of the tube had been scratched with a rod. The faintly yellow and rather soluble cuprous derivative was centrifuged and washed. It was found possible to avoid undue loss by interposing an aqueous alcoholic washing after every second washing with water. The water-alcohol treatment consisted in suspending the material in a small volume of water and then adding about 2 vol. of alcohol. After more than 12 washings the liquor remained cloudy after centrifuging although it still gave a precipitate with HCl and $BaCl_2$. The cuprous compound was suspended at this stage in water and decomposed with H_2S. The cuprous sulphide was centrifuged and washed with water. The combined solution and washings were concentrated to a small volume under diminished pressure and transferred to a small basin. Crystallization set in as soon as the basin was scratched. The *p*H was adjusted to 4·5 by the addition of $5N$ ammonia and concentration continued over phosphoric oxide *in vacuo*. After a night at 0° the peptide was collected, washed with water and dried at 56° *in vacuo*. Yield 264 mg. By concentrating the mother liquor a quantity of less pure material was obtained.

The peptide formed colourless rectangular plates or prisms which decomposed sharply at 197°. (Found: C, 38·7; H, 6·20; N, 16·0, 16·1; S, 12·85; amide N, 5·28 %. $C_8H_{15}O_4N_3S$ requires C, 38·6; H, 6·02; N, 16·9; S, 12·85; amide N, 5·6 %.)

It is evident from the analytical data that this peptide was not quite pure. An attempt was made to purify it by recrystallization from water followed by aqueous alcohol. The product however proved on analysis to be less pure than the starting material. This result is less surprising when account is taken of the great lability of the amide nitrogen in this compound (Table II).

Glutaminyl compounds exhibit a tendency to lose ammonia and undergo cyclization to derivatives of pyrrolidone [Bergmann & Zervas, 1933]. Evidence that no such change had occurred during the preparation of glutaminylcysteine is provided by the results of the amide nitrogen determination. Free pyrrolidonecarboxylcysteine would itself give no amide nitrogen but would form an ammonium salt which might bear a superficial resemblance to glutaminylcysteine. The ammonium salt would however liberate ammonia when dissolved in an alkaline buffer solution and distilled *in vacuo* under the conditions for the determination of amide nitrogen but without previous heating in acid solution. Two experiments made to test this point showed that only a negligible amount of ammonia was liberated by the peptide under these conditions.

Optical rotation. Glutaminylcysteine. 15·3 mg. in 0·5 ml. of water had α_{5461} $-0·15°$ in a 0·5 dm. tube whence $[\alpha]_{5461} = -9·8°$.

Glutaminylcystine. On account of the insolubility of this substance the determination was made in acid solution. To a solution of glutaminylcysteine (12·6 mg.) in water containing the theoretical quantity of hydrogen peroxide (1·11 ml.) was added HCl (0·25 ml. of 0·25 *N*), containing a trace of copper sulphate. When oxidation was complete the solution had α_{5461} $-0·55°$ in a 0·5 dm. tube giving $[\alpha]_{5461} = -119°$.

Iodine titration. The peptide (9·8 mg.) was dissolved in 5 % potassium iodide solution (5 ml.) and treated with approximately 0·01 *N* iodine solution (5·0 ml.). The excess of iodine was back-titrated with 0·01 *N* sodium thiosulphate. 4·68 ml. of 0·01 *N* iodine were required as against the theoretical amount of 3·94 ml. The high result obtained in this titration may be compared with that obtained by Harington & Mead [1935] for glutamylcysteine in which the cysteine is linked in an analogous manner.

Determination of labile sulphur.

The apparatus was similar to that described by Abel & Geiling [1925]. Preliminary experiments showed that a rubber stopper, if purified by sodium hydroxide solution in the usual way, liberated no sulphur when new. After being in use for some time, however, it became responsible for a large blank. The apparatus finally employed was therefore made entirely of glass.

Control of method. A freshly prepared solution of H_2S in ice-cold, oxygen-free NaOH was standardized by the procedure described by Treadwell & Hall [1919]. Portions (2·0 ml.) of this solution were boiled with water (3 ml.) and 0·2 *N* Na_2CO_3 (5 ml.) in the 30 × 150 mm. pyrex tube of the apparatus. A slow stream of nitrogen passed through the solution and reflux condenser and escaped through ammoniacal cadmium chloride solution contained in a 100 ml. conical flask. When 45 min. had elapsed, 5 ml. of approximately 5 *N* HCl were added to the sulphide solution through a tap funnel and ebullition was continued in a more rapid stream of nitrogen for 30 min. The receiving flask was then disconnected, the contents were diluted with water (25 ml.) and the cadmium sulphide was dissolved by the addition of 5 *N* HCl which was immediately followed by an excess of 0·01 *N* alkaline potassium iodate solution containing potassium iodide. The excess iodine was ultimately titrated with 0·01 *N* sodium thiosulphate solution.

The standard sulphide solution contained 0·111 mg. S per ml. by direct titration. The results from three control experiments were 0·111, 0·113 and 0·111 mg./ml.

Determination of labile sulphur in peptides. The peptide (about 16 mg.) was weighed into a standard 10 ml. flask, dissolved in a small volume of water, and treated with the theoretical quantity of hydrogen peroxide in the presence of a trace of ferric ammonium sulphate. When oxidation was complete the solution was made up to 10 ml. Portions (2·0 ml.) of this solution were treated as described above.

Determination of labile sulphur in insulin. A solution of crystalline insulin was standardized by the determination of nitrogen on aliquot portions (Kjeldahl). The labile sulphur in 2 ml. of this solution was determined as described above with the exception that capryl alcohol was added in order to diminish the frothing. A blank experiment showed that the result was thereby unaffected.

Determination of the amount of ammonia liberated by glutaminylcystine and cystylglutamine during treatment with acid or alkali.

Standard solutions, containing approximately 1·5 mg. per ml. of glutaminylcysteine or cysteylglutamine were prepared freshly for each experiment. Three 2 ml. aliquots of peptide solution were measured into test-tubes and treated with the theoretical quantity of hydrogen peroxide and a trace of copper sulphate. Two portions were used for duplicate determinations and the third as a control for the oxidation.

Acid treatment. The oxidized peptide solutions were mixed with an equal volume of 0·2 *N* HCl and were heated in a boiling water bath for 3 hours. During this time the tubes were closed by stoppers provided with short lengths of capillary tube. Before analysis the solutions were almost neutralized by the addition of 0·4 *N* NaOH.

Alkaline treatment. The oxidized peptide solutions were mixed with an equal volume of *N*/15 NaOH. Each tube was closed by a stopper which carried a small drying tube containing glass beads moistened with *N* HCl (0·2 ml.). After 3 hours in a thermostat at 34° the beads were rinsed with water. The rinsings were added to the contents of the tube and the now slightly acid solutions were washed into the ammonia distillation apparatus. Control experiments with a standard solution of ammonium sulphate showed that no loss of ammonia was incurred by this procedure.

Distillation of ammonia. The apparatus resembled that described by Parnas & Heller [1924]. The steam supply was regulated automatically by means of a piece of capillary tubing in the steam inlet. The vacuum was similarly controlled by a capillary leak inserted into the connection between the pump and the vessel enclosing the receiver. A side-arm on this vessel permitted the tip of the condenser to be rinsed into the receiver. The water-cooled condenser was of glass and gave rise to a constant blank equivalent to 0·007 mg. N. Truncated monax test-tubes (18 × 90 mm.) served as receivers.

For a distillation a receiver containing 0·01 *N* HCl (2·00 ml.) was adjusted so that the tip of the condenser ended just beneath the surface of the acid. Phosphate-borate buffer (5 ml. of a solution containing 27·2 g. KH_2PO_4 and 9·55 g. $Na_2B_4O_7, 10H_2O$ in a litre) was added to the sample in the distillation flask and the latter was connected to the apparatus by a greased joint. Sodium hydroxide-borax mixture (3 ml. of a solution containing 50 g. $Na_2B_4O_7, 10H_2O$ in a litre of 0·5 *N* NaOH) was added through a side-arm on the steam supply tube and the apparatus was evacuated. Distillation was maintained for 3 min. with the end of the condenser dipping beneath the surface of the liquid in the receiver and for 1 min. with the receiver lowered. In all 5 ml. of distillate were collected. Steam was then shut off and air admitted. The tip of the condenser was rinsed and the contents of the receiver were treated with potassium iodide solution (2 ml. of 5 %) and potassium iodate solution (3 drops of 4 %). After 3 min. the iodine was titrated with 0·01 *N* sodium thiosulphate.

Blank estimations on all the reagents and also an analysis of a standard ammonium sulphate solution were made before every determination of ammonia.

Determinations of amide nitrogen.

Ovalbumin. The following determinations of the amide nitrogen content of ovalbumin were made in order to check the accuracy of the method. The ovalbumin, which had been recrystallized by the use of sodium sulphate, was kindly provided by Dr Neuberger.

Method of hydrolysis	Equivalents of amide N per molecule
20 % HCl at 100° 3 hours	26·1
0·2 *N* HCl at 100° 20 hours	24·6, 24·4

The most recently determined value is 24 equivalents per molecule [Shore *et al.*, 1935].

Insulin. The crystalline insulin, which was kindly provided by Dr D. A. Scott, contained 9·2 % of moisture and 0·85 % ash. Approximately 15 mg. portions were weighed into test-tubes and were dissolved in 5 *N* HCl (4 ml.). It was found to be more advantageous to add water first (0·73 ml.) and then HCl of constant B.P. (3·27 ml.). The insulin was allowed to dissolve completely after the addition of the first drop of acid and the remainder was then run in.

The tubes, which were closed by stoppers carrying micro-reflux condensers, were heated for varying periods in a boiling water-bath. For analysis, each solution was washed into the distillation vessel and treated with phosphate-borate buffer (5 ml.) and with 10 *N* NaOH (1·85 ml.). 5 drops of bromothymol blue were added followed by sufficient 0·4 *N* NaOH to give a greenish yellow tint similar to that given by the buffer and indicator alone. The flask was then attached to the apparatus, sodium hydroxide-borax mixture (3 ml.) was added and the ammonia distilled in the usual manner.

APPENDIX.

The preparation and properties of the following substances which may prove to be of use in peptide synthesis are described below.

Glycine benzyl ester hydrochloride.
N-Benzylcarbonylcystylglycine benzyl ester.
α-Benzyl ester of *N*-benzylcarbonyl-γ-glutamylglycine benzyl ester.
N-Benzylcarbonyl-β-aspartylglycine.
α-Benzyl-*N*-benzylcarbonyl-β-aspartylcystine ethyl ester.
γ-Methyl-*N*-benzylcarbonyl-α-glutamyl-*S*-methylcysteine benzyl ester.

Glycine benzyl ester hydrochloride. Glycyl chloride hydrochloride prepared from 25 g. of glycine by the method of Fischer [1905] was added slowly to an ice-cold mixture of benzyl alcohol (40 ml.) and ether (40 ml.). More ether was added while the product was crystallizing from the mixture.

After a night at 0° the substance was collected and washed with ether. The yield was 48 % of the theoretical.

For purification the crude material (15 g.) was dissolved in water (35 ml.). The solution was washed with ether in order to remove a trace of benzyl chloride, cooled in ice-salt treated with anhydrous potassium carbonate (20 g.) and extracted with ether at the centrifuge. The aqueous layer was made into a sludge with more potassium carbonate and extracted twice more with ether. The ethereal extracts were united, dried over anhydrous potassium carbonate in the presence of norite, and treated with dry gaseous HCl until separation of the product was complete. The substance was collected and washed with ether. Glycine benzyl ester hydrochloride forms long colourless prisms melting at 139–140°. (Found: N, 6·94; Cl, 17·6 %. $C_9H_{12}O_2NCl$ requires: N, 6·96; Cl, 17·6 %.)

N-Benzylcarbonylcystylglycine benzyl ester. Glycine benzyl ester hydrochloride (9·6 g.) in a small volume of water was cooled in ice-salt and treated with anhydrous potassium carbonate (10 g.). The free ester was extracted with three portions of ethyl acetate. The ethyl acetate solution was dried over sodium sulphate, cooled in ice-salt and treated with *N*-benzylcarbonylcystyl chloride from 20 g. of *N*-benzylcarbonylcystine. After standing for several hours at room temperature the product was collected and washed with ethyl acetate and with ether and dilute acid. It was recrystallized from *n*-propyl alcohol and formed large prismatic needles melting at 167–168°. (Found: N, 6·9; S, 8·03 %. $C_{40}H_{42}O_{10}N_4S_2$ requires: N, 6·98; S, 7·98 %.)

α-Benzyl ester of N-benzylcarbonyl-γ-glutamylglycine benzyl ester. A solution of glycine benzyl ester in ethyl acetate was prepared as described above from glycine benzyl ester hydrochloride (6·5 g.). The solution was cooled in ice-salt and was treated with the acid chloride obtained by the method of Bergmann *et al.* [1933] from α-benzyl-*N*-benzylcarbonylglutamate (5 g.). (This acid chloride has been obtained crystalline in the form of small needles.) After standing for several hours the solution was filtered from precipitated glycine benzyl ester hydrochloride, washed with dilute acid and with water, dried over sodium sulphate and concentrated to a small volume under diminished pressure. The product which was precipitated by the cautious addition of ligroin was recrystallized once from ethyl acetate-ligroin and then twice from ethyl alcohol. It formed cigar-shaped crystals melting at 104–105°. (Found: C, 66·8; H, 5·90; N, 5·45 %. $C_{29}H_{30}O_7N_2$ requires: C, 67·2; H, 5·79; N, 5·41 %.)

N-Benzylcarbonyl-β-aspartylglycine. The acid chloride prepared by the method of Bergmann *et al.* [1933] from *N*-benzylcarbonylaspartic acid α-benzyl ester (3·0 g.) was dissolved in dry chloroform (15 ml.). This solution and *N* NaOH (8·5 ml.) were added in parallel portions during 60 min. to a well stirred solution of glycine (0·63 g.) in *N* NaOH (8·5 ml.). Stirring was continued for 15 min. after the last addition and then the mixture was centrifuged. The aqueous layer was washed with ether, made acid to Congo red, saturated with ammonium sulphate and extracted thoroughly with ethyl acetate. The ethyl acetate solution was dried over sodium sulphate, concentrated to a small volume under diminished pressure and treated with ligroin. The oil which separated soon hardened. The product was collected and crystallized from acetic acid-benzene. *N*-Benzylcarbonyl-β-aspartylglycine forms fine needles melting with decomposition at 153° after sintering. (Found: C, 51·6; H, 5·17; N, 8·4 %. $C_{14}H_{16}O_7N_2$ requires: C, 51·9; H, 4·94; N, 8·6 %.)

α-Benzyl-N-benzylcarbonyl-β-aspartylcystine ethyl ester. The acid chloride prepared from *N*-benzylcarbonylaspartic acid α-benzyl ester (3 g.) was shaken with a suspension of cystine ethyl ester hydrochloride (1·56 g.) in dry pyridine (10 ml.) and cooled in ice-salt. After 4 hours at room temperature 2 vol. of chloroform were added and the solution was filtered from excess cystine ethyl ester hydrochloride. The filtrate was evaporated to a syrup under reduced pressure and was treated with successive portions of water until it solidified. The material was dissolved in hot ethyl acetate, precipitated by ligroin and then crystallized from *n*-propyl alcohol. It formed colourless needles melting at 160°. (Found: N, 5·7; S, 6·56 %. $C_{48}H_{54}O_{14}N_4S_2$ requires: N, 5·75, S, 6·57 %.)

γ-Methyl-N-benzylcarbonyl-α-glutamyl-S-methylcysteine benzyl ester. An ethereal suspension of *N*-benzylcarbonyl-α-glutamylcysteine benzyl ester was treated with an excess of diazomethane at 0°. The acid passed into solution with evolution of nitrogen. After 60 min. the solution was filtered from a small precipitate and evaporated to dryness. The residue crystallized from ethyl acetate-ligroin. M.P. 85–86°. (Found: C, 59·6; H, 5·92; N, 5·8 %. $C_{25}H_{30}O_7N_2S$ requires C, 59·8; H, 5·98; N, 5·6 %.)

SUMMARY.

The syntheses of glutaminylcysteine and cysteylglutamine have been described.

In view of the suggestion of Jensen & Evans [1934] these peptides have been tested for hypoglycaemic activity and compared in their chemical behaviour with insulin.

Neither peptide in reduced or oxidized form exhibited any hypoglycaemic activity. The peptides showed a general resemblance to insulin in respect of the lability of their amide nitrogen but differed from insulin in that their sulphur was much more stable towards alkali.

The time course of liberation of ammonia from insulin by 5*N* hydrochloric acid at 100° indicates the presence of about 34 amide groups per mol. From this value together with published titration data it is concluded that insulin contains about 30% of glutamic acid.

The preparation and properties of some intermediates for peptide synthesis are recorded.

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CCXXVIII. LIVER GLYCOGEN.

IV. MOLECULAR STRUCTURE OF GLYCOGEN FORMED AFTER INGESTION OF GALACTOSE BY FASTED RABBITS.

By DAVID JAMES BELL.

From the Biochemical Laboratory, Cambridge.

(*Received 23 July 1936.*)

This paper describes the application of Haworth's "end-group assay" method [Haworth & Percival, 1932] to the elucidation of the molecular structure of glycogen formed in rabbits' livers subsequent to the oral administration of galactose. The glycogen was methylated and then hydrolysed and the cleavage products were separated and identified by the author's procedure [Bell, 1935]. A yield of 6 % of 2:3:4:6-tetramethylglucose was obtained, corresponding with a molecular unit in the methylated glycogen of 18 glucose units.

The following table records the general results of the investigation which was carried out on two groups of rabbits, A 25 animals and B 10 animals. The experimental conditions were purposely different in the two groups.

Table I.

Group	A	B
Fasted for	24 hrs.	48 hrs.
Galactose fed	5 g./kg.	10 g. each
Time allowed for absorption	2 hrs.	4 hrs.
Tetramethylglucose % found after hydrolysis of methylated glycogen	6·0	6·2
Number of glucose units in glycogen molecule	18	18

The methoxyl contents (corrected, Bell [1935]) of the methylated glycogens, coupled with the amounts of tetramethylglucose found in the cleavage products, corresponded most closely with an octadecasaccharide having, in A, 54 of the available 56 OH groups methylated, and in B, 53 OH groups methylated. Table II shows detailed results of the separation of the cleavage products.

Table II.

Specimen of methylated glycogen	A	B
OMe %	45·6	44·8
Amount of material hydrolysed (g.)	15·00	12·28
Yield of tetramethylglucose (g.)	0·901	0·768
Yield of trimethylglucose (g.)	12·650	9·592
Yield of dimethylglucose (g.)	1·60	1·760
Recovery of cleavage products %	93·6	91·0

That the assay of the relative amounts of dimethyl- and trimethyl-glucose affords additional aid in estimating the chain-length of the glycogen is shown in Table III.

Table III.

Theoretical.

			No. of mols. of cleavage products			Ratios by weight	
No. of C_6 units in chain	No. of free OH groups	OMe %	X Tetra-methyl-glucose	Y Tri-methyl-glucose	Z Di-methyl-glucose	Y/X	Y/Z
18	1	46·0	1	16	1	14·1	16·0
18	2	45·4	1	15	2	13·1	7·4
18	3	44·9	1	14	3	12·2	4·6
Found A		45·6				14·0	7·9
Found B		44·8				12·5	5·4

Discussion.

Haworth [1935] reported that his research school had found that glycogen could apparently exist in two forms, one composed of 12, the other of 18 glucose units per molecule. Previously, Haworth & Percival [1932] and Bell [1935] had found 12 glucose units as the basic molecular structure of rabbit liver glycogen.

The formation of glycogen as a result of galactose feeding has been studied by a number of investigators, but always with an emphasis on the physiological aspects of the problem. Harding *et al.* [1934] showed that such glycogen in rats was composed solely of glucose units. This fact was confirmed in the present instance by Dr Stephenson, using the technique of Stephenson & Yudkin [1936]. Deuel *et al.* [1933] obtained some evidence that galactose feeding in rats led to the formation of a glycogen which was more slowly broken down or utilized than in normally dieted animals.

In the light of present knowledge, the formation of this new glycogen apparently can be attributed only to the process of galactose feeding. The author's previous investigation of the molecular structure of rabbit liver glycogen consisted in the examination of two samples of material each obtained from 12–20 animals. At the time of publication, the fact that the two groups of animals were supplied with carbohydrate under radically different conditions was not considered to be of significance, particularly as glycogens of identical molecular size were obtained from both groups. Stated briefly, one group received a carbohydrate-rich diet, largely carrots and sucrose, whilst the other animals, after fasting, received glucose by intravenous infusion through the ear-vein by the elegant technique of Bridge & Noltie [1935]. By the second method, rapid formation of large amounts of liver glycogen took place.

The formation of the 18-unit glycogen, therefore, would not seem to be the result of mere rapid storage of carbohydrate in the liver.

A further important fact has emerged from this work. Although investigation of the methylated glycogen showed that it had a molecular structure of 18 glucose units, examination of the glycogen itself revealed no significant differences from the 12-unit polysaccharide. It therefore follows that such properties of glycogen itself as lend themselves to examination afford no clue as to the chemical molecular structure [see Bell & Young, 1934; Bell & Kosterlitz, 1935; Oakley & Young, 1936]. The suggestion by Donhoffer & Macleod [1932] that more than one kind of liver glycogen exists therefore seems to warrant further investigation.

EXPERIMENTAL.

Preparation of the glycogen.

Group A. 25 rabbits, fasted 24 hours, were given galactose, 2·5 g./kg. body weight, by stomach tube. About 2 hours later the animals were killed, the livers excised and ground with ice-cold 5 % trichloroacetic acid. After centrifuging, the glycogen was precipitated by the addition of 1 vol. alcohol. Subsequent purification was effected by the procedure of Bell & Young [1934]; yield 20 g.

Group B. 10 large rabbits were fasted 48 hours and to each were then given 10 g. of galactose in concentrated solution by stomach-tube. After 4 hours the animals were deeply anaesthetized with "Numal-Roche" and the livers excised and extracted by boiling water; subsequent purification as for group A; yield 13·5 g. from 700 g. liver tissue.

Properties of glycogens A and B.

Aqueous solutions of both were rather difficult to free from traces of nitrogenous material. This was finally effected by centrifuging at 15,000 r.p.m. The purified solutions were rather more opalescent and had very slightly higher opacities than 12-unit glycogen but otherwise appeared similar and gave the same red-brown colour with iodine. The properties are tabulated below:

Table IV.

Specimen	A	B
$[\alpha]_D$ in H_2O ($c=0·5\%$)	+201°	+200°
Reducing power [Macleod & Robison, 1929]	1·2 %	1·1 %
Phosphorus, organic	Nil	Nil
Phosphorus, inorganic	Nil	Nil
Ash	Nil	Nil

Biochemical investigation of hydrolysis product of glycogen.

(With M. STEPHENSON.)

The carbohydrate, $[\alpha]_D+52°$ (in water), formed by aqueous acid hydrolysis of the glycogen "A" was investigated by a fermentation method. A strain of *S. cerevisiae* was used which, when grown on glucose broth, centrifuged and washed, failed to ferment galactose at a measurable rate [Stephenson & Yudkin, 1936].

The CO_2 eliminated per mg. carbohydrate was measured in the Barcroft manometer as described in the above paper. The total CO_2 eliminated from the unknown sugar was compared with that from pure *d*-glucose. As is shown below, the CO_2/mg. sugar corresponds very closely in the two instances:

Pure glucose	Hydrolysis product
µl.	µl.
165·9	162·0

Carrying out a parallel large scale experiment, the yeast-free liquors, after fermentation had ceased, were found to contain less than 0·02 % of reducing material (Macleod & Robison).

Acetylation.

This was carried out by the procedure of Haworth and Percival:

Table V.

Specimen	A	B
Amount of starting material (g.)	8·5	13
Yield of acetate (g.)	14	19
Yield %	93	82
$[\alpha]_D$ in $CHCl_3$ ($l=2$, $c=2\%$)	+170°	+170°
$COCH_3$ %	44·0	44·4

Methylation.

A. 26 g. acetate were treated according to Haworth & Percival. After 10 methylations and subsequent purification, 15·8 g. of methylated glycogen were obtained (OMe, 45·6 %. $[\alpha]_D^{22^\circ}$ +210°: in $CHCl_3$: $l=2$, $c=6$ %). The product possessed the appearance and properties described by Bell [1935].

B. 19 g. acetate gave, after 8 treatments, 12·50 g. (93 %) of methylated glycogen. (OMe, 44·8 %. $[\alpha]_D^{22^\circ}$ +207°: in $CHCl_3$: $l=2$, $c=2·1$ %.)

Hydrolysis by aqueous acid and separation of cleavage products.

The procedure followed was exactly as described by Bell [1935] except that isolation of the tetramethylglucose in a state of purity, owing to the different relative proportions of tri- and tetra-methylglucoses, required that a 5 % solution in chloroform of the crude product, obtained after the chloroform water partitions, should be washed four times with one-third of its volume of water.

A. 15·00 g. were hydrolysed giving:

2:3:4:6-Tetramethylglucose, 0·901 g.; n_D could not be measured owing to crystallization in refractometer. $[\alpha]_D^{20}$ in H_2O, +82·1°; M.P. 85–88°; mixed M.P. showed no depression; OMe, 52·5 %.

2:3:6-Trimethylglucose, 12·650 g.; $n_D^{20^\circ}$, 1·4758; $[\alpha]_D^{20^\circ}$ in H_2O, +70°; M.P. 113–117°; mixed M.P. showed no depression; OMe, 41 %.

Dimethylglucose, 1·60 g; n_D^{20} 1·4860; OMe, 30·0 %.

B. 12·40 g. were hydrolysed. A small residue of partly hydrolysed material, 0·120 g., was isolated [see Bell, 1935]. This was deducted from the amount of starting material, making 12·28 g. The following fractions were obtained:

2:3:4:6-Tetramethylglucose, 0·768 g.; n_D^{20} 1·4570; $[\alpha]_D^{20^\circ}$ in H_2O +82°; OMe, 52·1 %; M.P. 85–88°; mixed M.P., no depression.

2:3:6-Trimethylglucose, 9·005 g.: $n_D^{20^\circ}$ 1·4740; $[\alpha]_D^{20^\circ}$ in H_2O, +70·7°; OMe, 41·5 %; M.P. 111–116°; mixed M.P., no depression.

"*Dimethylglucose*", 2·347 g.; n_D^{20} 1·4820; OMe, 32·2 %. The refractive index and methoxyl content indicate that the dimethyl-fraction here contains about 25 % of trimethylglucose. Correcting for this, the amounts of trimethylglucose and dimethylglucose become respectively, 9·592 and 1·760 g.

No trace of isomeric trimethylglucoses could be detected.

SUMMARY.

Glycogen formed in rabbit livers after ingestion of galactose has been shown to have a chemical molecular magnitude corresponding to 18 glucose units, the normally formed glycogen so far investigated being built up of 12. The new type of glycogen was obtained from two groups of animals, 35 in all.

The author thanks Sir F. G. Hopkins for his interest and encouragement. He is grateful to Dr Hans Kosterlitz for the crude material "A", to Miss M. Stephenson for carrying out fermentation experiments, to Mrs C. Lutwak-Mann for performing the phosphorus determinations and to the Technical Staff of this Laboratory for their willing assistance. Part of the expenses of the investigation was defrayed by the Medical Research Council.

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CCXXIX. STUDIES ON AMINO-ACID DEHYDROGENASE.

II. ACTIVATOR OF PROLINE DEHYDROGENASE.

By NALIN BANDHU DAS.[1]

From the Biochemical Institute, University of Stockholm.

(*Received 6 July 1936.*)

In a previous communication [Das, 1936] it was shown that there was present in pig kidney a thermostable substance capable of acting as activator of proline dehydrogenase. The present investigation was undertaken with the twofold object of studying the chemistry of the activator and the occurrence of the activator in yeast. Baker's yeast has been found to be a rich source of the activator. In the present investigation *dl*-proline has been used throughout as substrate.

Extraction of the activator.

In the earlier paper [Das, 1936] the activator was demonstrated in the enzyme solution from pig kidney which had been heated at 80° and *p*H 8·0 for 5 min. In the present investigation, in order to ascertain if it were possible to obtain the activator from baker's yeast, 100 g. of the latter (about 80 % moisture) were heated with 200 ml. water at 85° and centrifuged after cooling. The centrifugate was found to activate proline dehydrogenase considerably even in small amount (15·4 mg. solid). It was also proved that the activation was not due to the presence of a donator or to non-enzymic oxidation of proline, Table I.

Table I.

No.	Enzyme solution ml.	Yeast activator ml.	Phosphate buffer ml.	*M* proline ml.	μl. O_2 uptake after min. 15	30	45	60
1	1·0	—	1·0	0·1	26·2	57·4	89·1	115·3
2	1·0	0·5	1·0	0·1	87·1	161·8	214·7	249·0
3	1·0	0·5	1·0	—	—	—	—	—
4	—	0·5	1·0	0·1	—	—	—	—

Method of experiment.

The oxidation experiments were carried out in the Warburg-Barcroft apparatus as described in the previous communication [Das, 1936]. The enzyme solution was prepared as already described.

Purification and chemical nature of the activator.

In order to study the chemical nature of the activator attempts were made to obtain it in a relatively concentrated form by methods of precipitation and adsorption. Experimental details are given below and the results are summarized in Table II.

It was found that the activator could be adsorbed on animal charcoal, frankonit and acid clay from slightly acid solution and eluted by treatment with

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[1] Lady Tata Memorial Scholar.

Table II.*

Enzyme (*p*H 7·8) (ml.)	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
Phosphate buffer (*p*H 7·8, *M*/20)...	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
Proline, *M*	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1
3 experiments:									
Y (16)	—	0·5	—	—	—	—	—	—	—
Y (16) a	—	—	0·5	—	—	—	—	—	—
Y (47)	—	—	—	—	0·5	—	—	—	—
Y (48)	—	—	—	—	—	0·5	—	—	—
Y (67)	—	—	—	—	—	—	—	0·5	—
Y (68)	—	—	—	—	—	—	—	—	0·5
O_2 uptake (μl./60 min.)	75·8	89·7	80·5	188·0	242·7	141·5	135·4	416·7	206·3
Y (7)	—	0·5	—	—	—		—	—	—
Y (8)	—	—	0·5	—	—	—	—	—	—
Y (9)	—	—	—	0·5	—	—	—	—	—
Y (84)	—	—	—	—	—	0·5	—	—	—
Y (33)	—	—	—	—	—	—	—	0·3	—
Y (37)	—	—	—	—	—	—	—	—	0·2
O_2 uptake (μl./60 min.)	129·4	242·0	248·2	266·5	227·9	291·4	99·1	250·7	242·4
2 experiments:									
Y (82)	—	0·5	—	—	—	—	—		
Y (83)	—	—	0·5	—		—	—		
Y (82 + 83)	—	—	—	(0·5 + 0·5)	—	—	—		
Y (56)	—	—	—	—	—	0·4			
Y (61)	—	—	—	—	—	—	0·6		
O_2 uptake (μl./60 min.)	235·1	189·0	220·0	230·4	198·1	254·5	262·3		
Y (21)	—	0·5	—	—	—	—	—	—	
Y (26)	—	—	0·5	—	—	—	—	—	
Y (25)	—	—	—	0·5	—	—	—		
Y (101)	—	—	—	—	—	0·5	—	—	
Y (102)	—	—	—	—	—	—	0·5	—	
Ash of Y (101)	—	—	—	—	—	—	—	5 mg.	
O_2 uptake (μl./60 min.)	53·5	105·3	97·3	60·5	160·0	248·0	249·0	155·4	
Proline, *M*/2	0·1	0·1	0·1	0·1	—	—	—		
Proline, *M*	—	—	—	—	0·1	0·1	0·1		
Y (95)	—	0·5	—	—	—	—	—		
Y (97)	—	—	0·5	—	—	—	—		
Y (100)	—	—	—	0·5	—	—	—		
Y (37)	—	—	—	—	—	0·2	—		
Y (42)	—	—	—	—	—	—	0·2		
O_2 uptake (μl./60 min.)	111·5	192·5	198·4	177·4	97·8	241·7	80·7		

* For description of different fractions see the experimental section.

alkali from acid clay and frankonit but not from animal charcoal. The loss was considerable in the case of frankonit. Attempts to adsorb the activator on kaolin and aluminium hydroxide *Cγ* (Willstätter) had failed, the activity always remaining in the filtrate. Abortive attempts were also made to adsorb the activator on aluminium oxide (Merck, standardized according to Brockmann) in a "chromatogram" tube.

The activator was precipitated by salts of heavy metals such as mercury and silver. Attempts were made to precipitate with lead acetate but the activity was found to be missing both from the filtrate and the precipitate. Possibly the activator was adsorbed by lead sulphide. The filtrate and precipitate combined also failed to activate proline dehydrogenase.

It is probable that the activator is not dependent on the presence of a primary amino-group for it is not inactivated by nitrous acid. At *p*H 5·0 the activator is not affected in 8 hours by a current of air at 20° nor is it destroyed by H_2O_2.

Attempts to extract the activator by ether from alkaline solution failed and the whole of the activity was precipitated by alcohol. The activator was destroyed by ashing.

Influence of lactoflavin.

Lactoflavin was incapable of activating proline dehydrogenase showing that the activation was not due to the presence of flavin in the yeast extract (Table III).

Table III.

No.	Enzyme solution ml.	Lactoflavin (40 γ/ml.) ml.	Phosphate buffer ml.	*M* proline ml.	μl. O_2 uptake after min. 15	30	45	60
1	1·0	—	1·0	0·1	43·6	70·3	94·5	111·5
2	1·0	0·5	1·0	0·1	29·2	58·3	84·5	99·1
3	1·0	0·5	1·0	0·1	34·2	59·1	84·0	96·5

Influence of flavin-enzyme.

Formerly it was shown [Das, 1936] that flavin-enzyme [Warburg & Christian, 1932, 1, 2] failed by itself to activate proline dehydrogenase. The possibility was next considered that flavin-enzyme might act in combination with the activator to catalyse the oxidation of proline by the enzyme; this was however not the case (Table IV).

Table IV.

No.	Enzyme solution ml.	Heated enzyme solution ml.	Flavin-enzyme 0·2 g. in 10 ml. water ml.	Phosphate buffer ml.	*M* proline ml.	μl. O_2 uptake after min. 15	30	45	60
1	1·0	—	—	1·0	0·1	39·6	92·4	132·0	163·7
2	1·0	—	0·5	1·0	0·1	32·1	75·8	116·6	145·7
3	1·0	0·5	—	1·0	0·1	72·7	140·4	188·0	225·6
4	1·0	0·5	0·5	1·0	0·1	77·6	150·3	206·0	249·7

EXPERIMENTAL.

Preparation of fractions.

1. *Y (16) a.* 50 ml. yeast extract, prepared as described above, were shaken with 5 g. of animal charcoal (Merck) at *p*H 5·0 and left in the refrigerator overnight. Removal of the charcoal by filtration yielded solution Y (16) a.

Y (16). The charcoal residue was eluted by grinding with 24 ml. phosphate buffer (*M*/20, *p*H 7·8) and 1 ml. 1 % Na_2CO_3 for 30 min. and filtered. 25 ml. of clear solution were obtained.

2. *Y (48).* 40 ml. yeast extract were stirred with 2 g. frankonit at *p*H 5·0 for 30 min. and filtered.

Y (47). The frankonit residue was eluted by stirring with 16 ml. phosphate buffer (*M*/20, *p*H 7·8) and 4 ml. 1 % Na_2CO_3 for 30 min. and filtered.

3. *Y (68).* 100 ml. yeast extract were agitated with 5 g. acid clay at *p*H 5·0 for 30 min. and filtered.

Y (67). The acid clay residue was eluted by stirring with 20 ml. 1 % Na_2CO_3 for 30 min. The clear filtrate measured 25 ml.

4. *Y (8).* 50 ml. yeast extract [Y (7)] were adsorbed with 2 g. kaolin for 120 min. and centrifuged. The clear centrifugate formed fraction Y (8).

5. *Y (9).* 50 ml. yeast extract [Y (7)] were agitated with 10 ml. suspension of aluminium hydroxide Cγ (Willstätter) for 120 min. and centrifuged. The clear centrifugate formed fraction Y (9).

6. *Y (84)*. 50 ml. yeast extract were completely precipitated with 10% mercuric acetate. The precipitate was washed, suspended in water acidulated with acetic acid and decomposed with H_2S. The clear filtrate [Y (84)] was freed from H_2S by a current of air.

7. *Y (82)*. 100 ml. yeast extract were precipitated with saturated lead acetate at *p*H 5·0 and centrifuged. The precipitate was washed, suspended in water acidulated with H_2SO_4 and decomposed with H_2S. The clear filtrate [Y (82)] was freed from H_2S by a current of air and by distillation *in vacuo*.

Y (83). The filtrate from the lead acetate precipitate was freed from lead by passing H_2S and excess of the latter was removed by aeration and vacuum distillation. The final fraction measured 90 ml.

8. *Y (61)*. 10 ml. yeast extract [Y (56)] were passed twice through aluminium oxide (Merck, standardized according to Brockmann) in a "chromatogram" column at *p*H 5·0. The clear filtrate measuring 15 ml. formed fraction Y (61).

9. *Y (37)*. 20 ml. yeast extract [Y (33)] were completely precipitated with 10% $AgNO_3$ and centrifuged. The silver precipitate was decomposed with HCl and centrifuged. The clear centrifugate measuring 5 ml. formed fraction Y (37).

10. *Y (25)*. 50 ml. yeast extract [Y (21)] were made alkaline (*p*H 10·0) with NaOH and shaken three times with ether, each time with about 10 ml. The ethereal layer was evaporated to dryness and taken up in 10 ml. water.

Y (26). The aqueous layer after ether extraction and freeing from excess of ether formed fraction Y (26).

11. *Y (95)*. 200 g. baker's yeast were extracted as usual with 400 ml. water and the extract was completely precipitated with 10% mercuric acetate solution. The washed precipitate was suspended in water acidulated with acetic acid and decomposed with H_2S. The clear filtrate was freed from H_2S by aeration. This solution, measuring 40 ml., was then agitated twice with 2 g. portions of acid clay and was filtered under suction. The acid clay adsorbate was eluted twice with 10 ml. portions of 1% Na_2CO_3 solution; the eluate measuring 20 ml. formed fraction Y (95).

12. *Y (97)*. 2 ml. Y (95) were treated with 0·5 ml. "perhydrol" (Merck, "perhydrol" 1 vol. and 9 vol. water = 10 vol. 3% H_2O_2), warmed at 40° for 15 min. and then evaporated to dryness in a vacuum desiccator; the residue was taken up in 2 ml. water.

13. *Y (100)*. 2 ml. Y (95) were treated with 5 drops of glacial acetic acid and 5 drops of 25% $NaNO_2$ solution and heated at 90° for 5 min. Presence of excess of HNO_2 was verified by testing with starch-iodide. The solution was then evaporated in a vacuum desiccator and the residue taken up in 2 ml. water.

14. *Y (102)*. 20 ml. yeast extract [Y (101)] were aerated for about 8 hours at room temperature (20°) at *p*H 5·0.

15. *Y (42)*. 1·5 ml. Y (37) [silver fraction] were precipitated with alcohol and filtered, the residue being washed with more alcohol. The clear filtrate together with the washing was evaporated to dryness under reduced pressure and the residue taken up in 1·5 ml. water.

16. 10 ml. yeast extract [Y (101)] were burnt to ash. Total weight of the ash was found to be 14·8 mg.

SUMMARY.

1. An activator of the proline dehydrogenase of pig kidney has been found in baker's yeast.

2. The activator can be adsorbed on acid clay, frankonit and animal charcoal in slightly acid solution and can be eluted by alkali from acid clay and frankonit. The loss is considerable in the case of frankonit. Attempts to elute the activator from animal charcoal by phosphate buffer (*p*H 7·8, *M*/20) and alkali have failed. The activator is not adsorbed on kaolin, aluminium hydroxide $C\gamma$ (Willstätter) and on Al_2O_3 in a "chromatogram" column.

3. The activator can be precipitated by silver nitrate and mercuric acetate.

4. The activator is not inactivated by HNO_2, H_2O_2 or by aeration.

5. The activator is not extracted by ether from alkaline solution and is precipitated by alcohol.

6. Lactoflavin is incapable of activating proline dehydrogenase.

7. Flavin-enzyme is incapable of catalysing the oxidation of proline by the enzyme together with the activator.

The author is grateful to Prof. Hans von Euler for his interest in this work.

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CCXXX. THE SEPARATION OF GLYOXALASE ACTIVITY AND GLYCOLYTIC ACTIVITY BY MEANS OF RADIUM RADIATION.

By HERBERT GRACE CRABTREE.

From the Imperial Cancer Research Fund, London.

(Received 16 July 1936.)

Investigations of the changes induced in the metabolism of cells under the action of radium or X-radiation have been few, and have led to conflicting results. Differences of technique are probably responsible for this lack of harmony. Radiation has been applied to cells or their products existing in four different conditions: as extracts, surviving slices, cultures *in vitro* or intact in the organism. Whether the results obtained by each of these methods are really comparable is an open question.

Certainly cell extracts containing potent enzymes are relatively insensitive to radiation. Hussey & Thompson [1923–26], using radium emanation, reported a destructive action on the hydrolytic enzymes trypsin, pepsin and invertase. Willcock [1906] found tyrosinase insensitive to large doses of β-radiation. Crabtree [1932] and Havard [1935] showed that succinoxidase partially lost its activity after heavy radiation, but Havard [1934, 1935] found indophenol oxidase, lactate, citrate and glucose dehydrogenases quite insensitive. It seems doubtful if any conclusion as to the mode of action of radiation on the intact cell can be drawn from a study of its effects on such isolated systems.

Using surviving tissue slices Frick & Posener [1926] described a checking effect of X-rays on the anaerobic glycolysis of rat and mouse tumours, fowl embryos and rat testes, and an inhibition of the Pasteur effect in the case of rat testes. Crabtree [1932] found that tissues under constant irradiation *in vitro* gradually lose their power of using oxygen during a period when glycolytic processes remain unimpaired. This finding, that respiration is more vulnerable to radiation than glycolysis, was confirmed in a series of papers [Crabtree & Cramer, 1934, 1, 2; Crabtree, 1934] where the possibility of varying the sensitivity of tumour tissue to radiation by interference with the respiratory system is described. Later Crabtree [1935] showed that the relative vulnerabilities of these two energy-yielding processes depended on the temperature of the tissue at the time of radiation; at body temperature respiration was primarily affected, at low temperature glycolysis could be inhibited almost completely whilst respiration was unaffected. It was suggested that the differential damaging of tumour cells at low temperature by way of their characteristic glycolytic process might be of clinical value.

Holmes [1933, 1935], using cultures *in vitro* of embryonic rat kidney, found that γ-radiation inhibited their rate of breakdown of carbohydrate, but had little or no effect on the breakdown of protein. Under similar conditions no effect was detected on two special enzyme systems, arginase and lactic dehydrogenase, but the latter enzyme was more easily damaged in minced tissues of young than of old embryos.

Irradiation of normal tissues and transplanted tumours *in vivo*, followed by measurements of metabolism at suitable intervals have led to uncertain and divergent results. Adler [1930] irradiated rat testes with X-rays and radium and described the metabolic and structural changes induced. He found a progressive diminution of respiration with increasing tissue degeneration, accompanied by a corresponding rise of aerobic and anaerobic glycolysis. Löw-Beer & Reiss [1931], using X-rays, and measuring the metabolism of Jensen's rat sarcoma 1–4 days after irradiation, claim to have completely suppressed respiration without any detectable effect on anaerobic glycolysis or the Pasteur reaction. This contrasts sharply with the results of Crabtree [1932] who found some fall in respiration of the tissues of J.R.S. similarly irradiated with radium *in vivo*, but this was accompanied by the appearance of abnormally high aerobic glycolysis. These results were put forward with diffidence because of the difficulty of obtaining sections which had responded equally to the radiation damage and possessed an equal intensity of metabolism. Frick & Posener [1926] likewise were unable to obtain homogeneous slices and were sceptical of the significance of any results obtained by irradiation *in vivo*.

Since the simple procedure of lowering the temperature of tumour tissue during irradiation causes selective damaging of the glycolytic mechanism, leaving respiration unaffected, it was considered of interest to attempt to analyse this effect. It seemed possible that one stage of the reactions which occur in the breakdown of carbohydrate to lactic acid might be particularly sensitive to radiation, since the inhibition of glycolysis by irradiation at low temperature occurs more quickly and completely than any other biochemical process so far studied. Of the two principal schemes suggested as intermediary phases of carbohydrate breakdown, that of Neuberg, Dakin & Dudley involving methylglyoxal was more suitable for investigation, since the Embden-Meyerhof scheme was evolved from a study of tissue extracts which, owing to their insensitiveness to radiation, increase the technical difficulties to be overcome.

In this paper it is shown that radium radiation applied to highly glycolysing tissues at low temperature can suppress total glycolysis almost completely whilst glyoxalase activity is unaffected.

Methods.

After suitable irradiation of tissue slices, manometric measurements have been made of respiration (Q_{O_2}), anaerobic glycolysis ($Q_M^{N_2}$) and anaerobic glyoxalase activity. Q_{O_2} and $Q_M^{N_2}$ have the significance given in the original work of Warburg [1926], and anaerobic glyoxalase activity is similarly expressed as a function of dry weight of tissue, i.e.

$$\frac{\mu\text{l. } CO_2 \text{ evolved by lactic acid formation in } N_2}{\text{hours} \times \text{mg. dry tissue}}.$$

The methylglyoxal used was prepared by the method of Riley *et al.* [1932]. Fractional distillation at low pressure yielded a product containing less than 3 % acid and this was used in sugar-free bicarbonate media in a concentration of $0{\cdot}01\,M$. The results were similar whether the methylglyoxal was added to the Ringer-bicarbonate before placing the manometers in the thermostat, or tipped it from a side-bulb after equilibrium at 37·5° had been attained.

Experiments with untreated tissues.

Jowett & Quastel [1934] found that glyoxalase activity was proportional to the surface area rather than the weight of the tissue, being apparently determined by the small depth to which methylglyoxal diffuses into the slices. They were unable to determine if there was a limit to glyoxalase activity as the slice-thickness decreased. As a preliminary to the radiation experiments, the glyoxalase activities of a series of tissues, including many tumours, were measured in order to check this finding. The results confirm the work of these authors to a large extent, but it was found unnecessary to calculate the activity per unit area rather than per unit weight if the slices were cut as thin as possible, i.e. approximately 0·2 mm. thick. Since this approaches the limit of thinness of tissue-slices which are obtainable in practice with a free-handed razor, and consistent values were invariably found, it suggests that the maximum glyoxalase activity is closely approached in measurements on such thin slices. In the case of mouse tumours which generally contain necrotic patches, which on removal give healthy sections of irregular shape, the error introduced by measurement of the surface area must be very considerable, and hence the simpler procedure of weighing has been used throughout this work.

In Table I a series of typical results are collected.

Table I. *Glyoxalase activity of different tissues.*

Ringer's solution containing 0·025 *M* $NaHCO_3$ and 0·01 *M* methylglyoxal.
Temp. = 37·5°. Gas phase = 5% CO_2 in N_2.

Tissue	Time of measurement (min.)	Dry wt. of tissue (mg.)	Thickness of slice (mm.)	Surface area (both sides) (cm.^{2})	Anaerobic glyoxalase activity (based on wt.)	Anaerobic glyoxalase activity (based on surface area)
Rat liver	60	2·08	0·21	1	101·0 → 76·2	210 → 158
	,,	4·11	0·41	1	46·0 → 31·0	189 → 128
	,,	6·20	0·62	1	31·3 → 27 6	194 → 171
Rat liver	45	2·01	0·2	1	67·8 → 46·1	136 → 92
	,,	4·41	0·22	2	73·5 → 44·1	162 → 96
	,,	7·48	0·25	3	61·2 → 48·2	152 → 119
Rat kidney	60	2·34	0·23	1	72·7 → 46·3	170 → 108
	,,	2·18	0·22	1	76·1 → 40·2	166 → 87
	,,	3·34	0·33	1	52·7 → 30·0	176 → 100
	,,	5·92	0·59	1	36·1 → 19·4	214 → 115
Rat spleen	60	3·00	0·30	1	59·2 → 42·1	178 → 126
	,,	3·18	0·32	1	54·0 → 40·9	172 → 131
	,,	4·93	0·49	1	54·3 → 36·0	208 → 178
	,,	6·01	1·20	0·5	16·7 → 11·0	200 → 132
Rat pancreas	45	7·09	—	—	6·9 → 5·9	—
	,,	14·28	—	—	15·1 → 10·6	—
	,,	12·55	—	—	9·7 → 7·3	—
	,,	33·22	—	—	7·6 → 6·5	—
Jensen's rat sarcoma	60	2·01	0·20	1	40·5 → 38·1	81 → 76
	,,	1·93	0·19	1	40·9 → 36·1	79 → 70
	,,	4·30	0·43	1	20·1 → 16·1	86 → 69
	,,	6·30	1·26	0·5	16·4 → 11·4	103 → 71
Jensen's rat sarcoma	90	2·06	0·21	1	34·1 → 29·6	70 → 61
	,,	2·82	0·28	1	28·6 → 25·3	81 → 72
	,,	3·85	0·38	1	21·6 → 17·3	83 → 66
Tar carcinoma 2146	30	2·06	0·21	1	41·9 → 38·3	87 → 79
	,,	1·81	0·18	1	47·2 → 44·0	85 → 79
Crocker sarcoma	30	1·95	0·20	1	44·6 → 43·2	87 → 84
	,,	2·01	0·20	1	41·8 → 40·4	84 → 81
37 sarcoma	30	1·96	0·20	1	40·7 → 30·5	80 → 60
	,,	2·05	0·21	1	38·4 → 31·2	79 → 64
Glycogen carcinoma 113	30	2·56	0·26	1	30·9 → 26·2	79 → 67
	,,	2·68	0·27	1	32·6 → 25·5	87 → 68
Adeno-carcinoma 27	30	2·62	0·26	1	38·3 → 29·1	100 → 76
	,,	2·41	0·24	1	43·5 → 32·5	105 → 75
Tar carcinoma 173	30	1·89	0·19	1	28·2 → 25·6	53 → 48
		2·10	0·21	1	33·5 → 28·1	70 → 59

→ indicates that the values were not constant during the time of measurement, only the initial and final values being given.

In the case of normal tissues the degree of activity is of the order found by other workers and no comment is necessary except in the case of pancreas. This tissue cannot be satisfactorily sliced owing to its soft consistency, and the measurements were made on relatively thick masses, with the ratio surface area/weight of a low and incalculable order. Yet a very considerable activity is shown, the anti-glyoxalase obtained from extracts of pancreas apparently not functioning, or at any rate ineffectively when the cells are intact. In the case of tumour tissues Jowett & Quastel [1934] found the activity of glyoxalase to be of the same order as that of total glycolysis. This contrasts with the results of Platt & Schroeder [1934] who reported very low glyoxalase activity in Philadelphia sarcoma No. 1, and Walker carcinoma No. 256. The results with mouse and rat tumours included in Table I are in agreement with those of Jowett & Quastel, showing a high glyoxalase activity in every tumour strain measured, and moreover being almost identical with the high total glycolysis characteristic of transplantable tumours. Platt & Schroeder see no evident relationship between the high glycolysis of tumour tissue and the low glyoxalase activity which they found. If the rate of transformation of methylglyoxal into lactic acid determines the high rate of glycolysis in tumour tissues, the results given here are not inconsistent with the conception that glyoxalase participates in the glycolytic process.

A noticeable feature was the different behaviours in the change of reaction rate of normal and tumour tissues. Jowett & Quastel record a rapid fall in glyoxalase activity during incubation, and a similar effect was observed in these experiments with normal tissues. But tumour tissues, in most cases, retained approximately their initial activity for long periods, in two experiments up to 5 hours.

Experiments with irradiated tissues.

The technique of irradiation has been previously described [Crabtree, 1935]. In order to obtain effects as quickly as possible, mixed $\beta+\gamma$ radiation was used from two applicators each containing 110 mg. of $RaBr_2, 2H_2O$ spread over an area 23 mm. square. Radiation was applied at low and body temperatures for suitable periods, after which the respiration (in phosphate buffer), anaerobic glycolysis and glyoxalase activity were measured. Some typical results obtained with tumour tissues are collected in Table II.

Irradiation of tumour tissue at body temperature for 4–5 hours has no damaging effect on the glycolytic or glyoxalase systems. Irradiation at low temperature largely suppresses total glycolysis, but leaves glyoxalase activity unimpaired. Respiration of tissues irradiated at body temperature was not measured, since previous work had shown that effects on respiration are only detectable with certainty after the lapse of a longer period than 4–5 hours.

The dose required to produce a large inhibition of total glycolysis can be considerably lessened by the method of divided doses over 5 hours. With the radiation intensities available it has been found impossible to shorten the period during which this effect is produced, but applications of radiation during the first, third and fifth hours, with the tissue at low temperature throughout, produce an effect of similar magnitude to that following continuous irradiation. Platt & Schroeder [1934] emphasize the necessity of maintaining the co-enzyme glutathione in adequate concentration during measurements of glyoxalase activity in extracts. It is clear that the prolonged treatment *in vitro* with an irrigating artificial saline medium and heavy radiation have no effect on cell glutathione, since glyoxalase activity remains constant for many hours and is not enhanced by the addition of glutathione.

Table II. *Respiration, glycolysis and glyoxalase activity of tumour tissue after irradiation at low and body temperatures.*

Tumour	Time of irradiation t_r (hr.)	Temp. during irradiation ° C.	Control						Irradiated		
			Q_{O_2} (phosph.)		$Q_M^{N_2}$		Anaerobic glyoxalase activity				
			Initial	After t_r	Initial	After t_r	Initial	After t_r	Q_{O_2} (phosph.)	$Q_M^{N_2}$	Anaerobic glyoxalase activity
Jensen's rat sarcoma	4	0–5	8·2	7·0	34·3	35·6	30·2	31·0	6·8	8·6, 11·2	28·3, 30·6
,,	4	,,	8·0	8·2	29·3	26·4	27·6	28·3	7·6	7·1	25·9, 30·6
,,	5	,,	10·2	8·9	38·9	35·6	27·9	29·3	8·0	5·1	28·0, 24·9
,,	5	,,	9·3	9·6	30·6	31·0	26·0	29·0	7·9	6·0	30·1, 25·3
,,	5	,,	6·4	7·0	37·0	35·1	30·2	26·8	8·0	3·4	24·0, 33·0
,,	5	,,	11·8	9·0	34·0	30·2	24·0	30·2	9·2	8·3	28·4, 27·5
,,	5	,,	10·0	9·1	36·0	33·0	32·3	30·0	8·7	4·9	35·0, 27·1
,,	4	37·5	—	—	41·2	42·0	26·3	28·2	—	36·0	26·8, 30·5
,,	4	,,	—	—	36·3	33·1	34·7	30·6	—	29·3	28·6, 33·4
,,	5	,,	—	—	30·2	31·6	33·5	26·0	—	29·4	26·5, 28·0
,,	5	,,	—	—	40·4	35·4	35·8	30·0	—	35·1	30·0, 24·8
Adeno-carcinoma 27	4	0–5	14·1	12·6	30·1	31·3	38·4	36·5	11·8	13·1	33·6, 30·2
,,	5	,,	10·0	11·1	32·6	30·0	34·6	35·0	9·4	7·1	33·0, 28·9
,,	5	,,	9·6	9·3	35·4	33·0	30·3	28·1	11·3	5·0	34·0, 30·9
37 sarcoma	4	,,	14·6	12·3	34·6	33·0	34·0	36·1	12·6	6·6	33·7, 33·8
,,	5	,,	10·2	11·6	40·1	38·6	36·3	35·2	10·6	4·2	34·0, 35·1
,,	5	,,	11·9	9·9	43·2	41·4	34·1	35 1	11·0	3·8	32·0, 36·8

That the glyoxalase activity of tumour tissue is not exceptional in its insensitivity to radiation, is apparent from results obtained by irradiating some normal tissues. Table III shows that irradiation at low temperature with large doses fails to affect the respiratory system and has little effect on glyoxalase. In the case of liver, and to a less extent of testes, the experimental conditions alone cause a noticeable fall of glyoxalase activity. The added effect of radiation is negligible except in one case of kidney tissue irradiated for 6 hours. Whether the glyoxalase activity which then falls spontaneously in liver tissue can be restored by added glutathione was not tested.

Table III. *Respiration and glyoxalase activity of some normal tissues after irradiation at low temperature.*

Rat tissue	Time of irradiation at 0° C. t_r (hr.)	Control				Irradiated	
		Q_{O_2} (phosph.)		Anaerobic glyoxalase activity			
		Initial	After t_r	Initial	After t_r	Q_{O_2} (phosph.)	Anaerobic glyoxalase activity
Kidney	4	12·3	14·1	73·3	68·8	12·2	52·3, 46·4
,,	4	14·1	12·3	59·6	63·6	11·1	54·5, 64·5
,,	6	15·1	13·6	62·7	62·7	16·1	38·2, 34·1
Liver	4	6·9	6·9	65·2	40·6	6·5	36·8, 40·0
,,	4	5·3	5·6	48·0	34·0	5·7	30·3, 26·4
,,	5	7·0	6·5	51·0	30·3	5·3	26·4, 28·1
,,	5	5·5	5·6	44·3	21·4	4·8	17·4, 25·2
Testes	4	9·3	9·0	27·6	20·5	9·1	19·6, 17·3
,,	5	9·2	8·5	24·2	22·5	8·8	17·3, 14·8
,,	6	8·6	8·7	23·8	18·4	8·2	15·0, 16·0

Some experiments were carried out with rat retinas to test if the above results with tumour tissues were true for another highly glycolysing tissue. This proved to be true, and it was found that retina was exceptionally sensitive to radiation. It is possible to obtain complete inhibition of glycolysis by irradiating retina at low temperature for only 2 hours, and 60–70% inhibition in one hour. This represents the most rapid effect as yet demonstrated on any biochemical process in living tissue. It is technically almost impossible to irradiate retinas at body temperature and then draw conclusions as to metabolic changes. As was shown by Crabtree [1934] retina is exceptionally sensitive to the damaging effect of bicarbonate media at 37°, the rapid fall of respiration and glycolysis making experiments with radiation of questionable significance. At low temperature, however, no metabolic change is obvious after irrigation with bicarbonate media for periods up to 4 hours. The general result was the same as with tumour tissues, a complete suppression of the sensitive total glycolysis leaving the insensitive glyoxalase system intact. Respiration again suffered no damage. Some results are given in Table IV.

Table IV. *Changes in the metabolism of rat retinas after irradiation at low temperature.*

	Control			Irradiated		
Time of irradiation (hr.)	Q_{O_2} (phosph.)	$Q_M^{N_2}$	Anaerobic glyoxalase activity	Q_{O_2} (phosph.)	$Q_M^{N_2}$	Anaerobic glyoxalase activity
4	—	65·8	84·5	—	6·3, 11·6	76·3, 80·2
4	15·9	56·3	67·2	13·3	7·5, 6·0	61·8, 56·3
3	—	68·1	82·5	—	11·6, 8·9	74·6, 78·3
3	—	49·3	86·4	—	5·4, 7·1	80·3, 74·9
2	—	47·8	86·8	—	3·2, 9·1	92·0, 84·0
2	20·9	77·1	78·3	18·1	4·5, 9·4	80·1, 73·6
2	—	67·4	82·3	—	7·3, 3·1	84·0, 74·6
2	23·6	55·4	85·0	24·2	6·0, 4·3	80·0, 78·0
2	—	60·2	76·3	—	3·2, 5·7	69·3, 79·1
1	—	62·0	68·9	—	18·5 → 5·8	69·4, 73·5
1	19·4	68·4	73·2	17·3	25·6 → 3·5	70·1, 67·8
1	—	89·3	96·1	—	27·3 → 7·3	86·2, 89·0

→ indicates that the values were not constant over the time of measurement (½ hr.). Initial and final values only are given.

DISCUSSION.

It has been shown that it is possible by means of radiation to separate various biochemical mechanisms in cells. Respiration and glycolysis, two energy-yielding processes normally allied in a relationship determined by the Pasteur effect are differentially attacked, respiration being more vulnerable at body temperature, glycolysis at low temperature. Glyoxalase activity, until recently conjectured to be essential as a step in the breakdown of hexose to lactic acid, is unaffected by radiation which almost completely suppresses total glycolysis. The conception that radiation produces a generalized damage to cells, leading to slow degeneration, can no longer be held, and the analysis of the varying vulnerability of fundamental biochemical processes seems a hopeful method of approaching the clinical problem of selectively sensitizing tumour cells in the host. Some workers have utilized the findings of metabolic changes induced *in vitro* as a basis of attempts to destroy tumours *in vivo* more readily. Moppett & Harker [1936] on the basis of experiments by Crabtree & Cramer [1934, 1, 2] injected sub-lethal doses of cyanides into living animals bearing transplanted tumours, and claimed that this treatment enhanced the sensitivity of tumours to radiation Franks *et al.* [1934] injected the maximum dose of iodoacetic acid which could be tolerated directly into transplanted tumours intact in the host and found that growth was inhibited and more regressions occurred, the inhibitions being more pronounced when this process was combined with radiation. For cases of superficial tumours, it seems more hopeful to make use of the suppression of glycolysis at low temperature as a basis of differential attack on the tumour.

Since glycolysis is extremely vulnerable at low temperature it would be reasonable, by analogy with the known effects of other inhibitors, to anticipate an attack on some link in the chain of reactions ending in lactic acid. If methylglyoxal occupies the prominent position in this chain which has been attributed to it, it is clear from the results recorded here that some previous stage, possibly the reaction involving phosphorylation of hexoses or the splitting of the hexose molecule to the 3-carbon stage, is the primary point of attack of radiation under the conditions used. Attempts to demonstrate this have led to no conclusion. Hexosephosphates are metabolised at a low level by tumour tissues *in vitro*. After irradiation, no detectable effect on the rate of this reaction was measurable, the low values found by other workers [Boyland & Mawson, 1934; Harrison & Mellanby, 1930] persisting.

The separation by radiation of the glycolytic and glyoxalase systems in intact cells is unlike the simultaneous effects produced by the well-known chemical inhibitors on these systems.

Meyerhof [1925] found that the glyoxalase activity of brain tissue was largely checked when the medium was saturated with heptyl alcohol, and compares this with the total checking of glycolysis of tumour tissues by the same agent. Fluoride and iodoacetic acid check both systems in varying degrees. Dickens [1933] emphasized the difference in the concentrations of iodoacetic acid required to check glyoxalase activity in crude and purified liver extracts, the dialysed extracts requiring a much lower concentration, of the same order as that required to produce the effect in liver slices. Some unsuccessful experiments were made during the course of this work to test the possibility of separating glyoxalase activity and glycolysis with different concentrations of iodoacetic acid. Iodoacetic acid poisoning is a time reaction, and though the rate of checking of glyoxalase activity was perhaps a little slower than that of total glycolysis, concentrations of 2 to $5 \times 10^{-4} M$ brought both reactions to a standstill in 20 min., and lower concentrations were ineffective in separating the two systems during the experimental period.

Jowett & Quastel [1934] used the fact that various chemicals and pancreatic extracts inhibit glyoxalase action and glycolysis in a similar manner as a supporting argument in favour of methylglyoxal occupying a key position in the main glycolytic process. The experiments outlined here show that argument to be invalid, and would harmonize equally well with the scheme of carbohydrate breakdown postulated by Embden & Meyerhof in which methylglyoxal plays no part.

The rapid fall of glyoxalase activity *in vitro* observed by previous workers encouraged the hope that irradiation at body temperature, or in the presence of inhibitors of respiration, might adversely affect glyoxalase activity in a selective manner and lead to the reverse way of separating it from glycolysis, but this was not accomplished. Glyoxalase activity survived intact in tumour tissue under constant irrigation with various media and heavy radiation over many hours.

SUMMARY.

1. The anaerobic glyoxalase activities of normal and tumour tissues have been measured. All tissues, including pancreas, show a considerable activity, and in the case of tumours this is high, and of the same order of magnitude as anaerobic glycolysis.

2. Rat retina, irradiated at low temperature, is the most sensitive tissue so far examined. Suppression of glycolysis can be effected in 1–2 hours.

3. Total glycolysis and glyoxalase activity can be almost completely separated by irradiation at low temperature, since the enzyme glyoxalase is insensitive to radiation.

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CCXXXI. CHOLESTEROL FEEDING AND FAT METABOLISM.

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The experiments to be described were undertaken as part of a study of the chemical changes produced in artificially induced fatty infiltrations and degenerations. Recent work has shown that feeding of cholesterol to animals brings about well marked pathological changes. The subject of arteriosclerosis and its possible relation to dietary cholesterol is dealt with in Cowdry's [1933] book and the later work is reviewed by Brody [1935]. The cholesterol "fatty liver" has also been the subject of much investigation and it is mainly with this aspect that the writer is concerned.

Cholesterol and growth.

(*a*) *Experiments with rats.* The animals used were drawn from the piebald laboratory stock. Litter-mates of approximately the same weight were taken soon after weaning and were 4–6 weeks old. The room in which the animals were confined was kept at a constant temperature. The diets fed to the animals were made up as shown in Table I.

Table I. *Rats' diets.*

All figures in g.

	Fat-free	Fat-free plus cholesterol	Normal synthetic	Normal synthetic plus cholesterol
Caseinogen	23	23	23	23
Rice starch	50	50	40	40
Cane sugar	22	22	17	17
Arachis oil	0	0	15	15
Salt mixture	5	5	5	5
Yeast	7·5	7·5	7·5	7·5
Cod liver oil	0	0	2·5	2·5
Radiostoleum	One drop per diem		0	0
Cholesterol (B.D.H.)	0	2	0	2
Calories per 100 g. food	354	347	434	426

Notes. The caseinogen used for the growth of rats shown in Fig. 1 was "Glaxo physiological caseinogen AB". This is heat-treated ashless caseinogen. In all subsequent experiments, Series B "Glaxo ashless casein E." was used. This is ashless caseinogen exhaustively extracted with alcohol to the maximum possible removal of ether-soluble matter. The salt mixture is No. 185 of McCollum & Davis [1915]. The yeast is a dried yeast preparation of trade name "Ceramealo".

The cholesterol was the normal commercial preparation (B.D.H.) and was not further purified. It was carefully mixed with the other constituents of the diet. No calorigenic value is given to the cholesterol but the cod liver oil is counted as dietary fat. The cholesterol intake of the rats is approximately 0·25 g. per rat per day.

Series A (Fig. 1) shows the growth curves obtained when the dietary source of caseinogen was the normal physiological caseinogen. Series B (Fig. 2) shows the result of using alcohol-extracted caseinogen. This type of caseinogen was substituted in the diets to ensure that the extraneous fats were minimum.

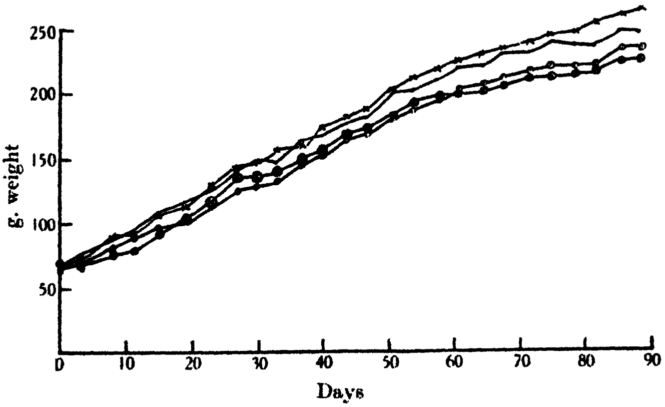

Fig. 1. Growth curves of animals fed on various synthetic diets plus 2 % cholesterol. The weights are the average of two males in each series. Series A using physiological caseinogen.
—·— Fat-free diet; ○-○-○ fat-free diet + cholesterol; ×-×-× normal synthetic; ●-●-● normal synthetic + cholesterol.

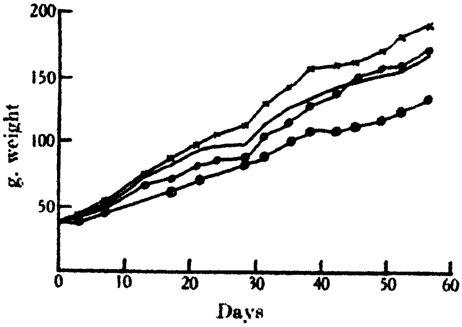

Fig. 2. Series B using alcohol-extracted caseinogen. The weights are the average of four males in each series. Markings as for Fig. 1.

The males of series A alone are shown, but the female litter-mates were also fed and gave the same type of growth curves. The results for the liver and body fats of the females are included in Table III.

Table II gives the calorie intake of the animals and shows that the calorie intake of food on the diets is well matched except that in series B there is a significantly decreased intake for the animals on the normal synthetic diet plus cholesterol. It will be seen from the figures that the presence of cholesterol affects the growth rate in both cases but particularly so in series B. This point will be discussed later. The earlier part of the growth curves only is shown. Some of the animals in series A were actually fed over 130 days. The difference in the food intakes during the later periods between the control animals and those fed with cholesterol was not significant. It averaged about 60 calories per rat per day. In addition to the animals fed on the synthetic diets a series was put

Table II.

Food intake expressed as calories per rat per day on the various diets. The figures are average intakes over the period stated in the first column. For series A the diet of 2 male rats is averaged and for series B that of 4 males.

	Series A				Series B			
Period of feeding days	Fat-free	Fat-free plus cholesterol	Normal synthetic	Normal synthetic plus cholesterol	Fat-free	Fat-free plus cholesterol	Normal synthetic	Normal synthetic plus cholesterol
0–15	32·9	32·0	37·2	36·7	25·0	25·4	29·7	26·5
15–28	46·9	45·1	50·3	52·5	35·2	32·7	41·6	30·8
28–40	55·6	45·8	47·4	50·5	43·8	45·8	51·3	34·4
40–58	60·0	60·3	60·1	60·0	49·1	52·3	56·0	42·3
58–82	62·1	59·2	62·6	60·0	—	—	—	—

up on the normal stock laboratory diet. This consists of bread and milk with corn and greenstuff added. The animals grew well on this diet and the rate of growth of normal animals and those taking 2 % cholesterol in their diet is shown in Fig. 3. The difference in the curves is due only to the initial difference in the weights of the animals. The added cholesterol has little effect on the growth rate. Mature animals were also given the stock diet but there was no noticeable effect. Thus 4 mature females of average weight 230 g. fed for 64 days with a diet containing 2 % cholesterol showed an average increase in weight of 27 g. as compared with an increase in the weight of the controls of 20 g.

At the end of a feeding period the animals were killed by cutting their throats under light anaesthesia and collecting the blood which was estimated with the carcass. The liver was always weighed as far as possible free of blood. Autopsy of the animals revealed that the fatty liver was only to be found in those animals which were given the normal synthetic diet, i.e. that containing added fat. Those on the stock diet showed no obvious change, a fact that was borne out on analysis. It was also observed that the animals showing the "fatty liver" appeared to be generally in a poor state of nutrition. There was very evidently less body fat. The carcass was therefore analysed for its fat content by the method of Leathes & Raper [1925]. This method does not give any idea of the distribution of the lipin material but it serves as an admirable and fairly rapid method of estimating total fat. The fats are estimated as fatty acids and the cholesterol as the free alcohol together with any other unsaponifiable matter. The question of the distribution of the lipins is at present in hand but it is not intended to deal with it in this communication. The carcass was normally saponified directly, the fur being included. The animals were not gutted. About one-sixth the weight of the carcass of KOH added as 50 % solution was sufficient to saponify the carcass after heating on a water-bath for 2 hours, alcohol being added after about 1½ hours. The mixture was made up to volume and the fat estimated in an aliquot portion. The results obtained are shown in Table III. The figures for fat determinations after the food had been taken for varying times are shown for the stock diet. It will be seen that there is no very marked effect. Normally the animals on the synthetic diets had the remains of the previous day's food in the dishes. It is somewhat more difficult with synthetic diets than with the stock diet to ensure the animals feeding at definite times, the tendency being for frequent feeding. Table III shows that the cholesterol effect is not very marked until after about 60 days' feeding. The

Table III. *The fat contents of the livers and carcasses of control and cholesterol-fed rats.*

a is the control animal and *b* the cholesterol-fed. In some experiments two animals were pooled for analysis.

									Carcass	
Diet	No.	Sex		Days on diet	Wt. of animals g.	Av. wt. of livers g.	Liver as % body wt.	"Fat" in liver %	g. fat	As % of body wt.
Fat-free Series A	1	♀	*a*	85	158 170	7·5	4·5	0·32	37·0	11·85
			b		175 153	8·1	4·9	0·78	37·0	12·0
	2	♂	*a*	131	298 270	12·0	4·2	1·41	43·4	8·35
			b		267 260	11·3	4·3	1·33	38·8	7·40
Series B	1	♂	*a*	59	180 210	9·8	5·0	0·23	47·6	12·85
			b		186 197	9·6	5·0	0·54	36·8	10·25
	2	♂	*a*	87	180 158	7·2	4·2	2·93	42·2	13·2
			b		186 194	9·2	4·8	3·48	41·8	11·45
Normal synthetic	1	♀	*a*	89	152 188	8·2	4·8	0·21	43·2	13·4
			b		139 220	11·4	6·4	13·8	34·9	10·5
Series A	2	♂	*a*	107	302	12·4	4·1	1·11	—	—
			b		354	12·8	3·6	12·5	—	—
	3	♂	*a*	117	274	11·5	4·2	3·6	20·0	7·6
			b		237	12·6	5·3	12·7	6·3	2·7
Series B	1	♂	*a*	41	190	9·7	5·1	4·43	14·2	7·9
			b		132	7·8	5·9	8·23	4·9	4·0
	2	♂	*a*	63	230 216	10·2	4·6	1·13	64·6	15·4
			b		162 133	7·3	4·9	10·0	32·0	11·6
	3	♂	*a*	73	190	8·7	4·6	3·45	27·8	15·7
			b		123	6·0	4·9	5·25	9·1	8·7
Full diet (laboratory stock diet)	1	♂	*a*	64	378	17·6	4·7	2·5	54·2	15·35
			b		382	18·3	4·8	2·63	61·5	17·2
	2	♀	*b*	92	187 187	16·7	4·5	1·83	42·6	11·4
	3*	♀	*a*	66	167	7·3	4·4	2·98	—	—
			b		185	8·0	4·3	3·78	—	—
	4*	♀	*a*	66	192	6·9	3·6	3·52	—	—
			b		209	7·7	3·7	3·20	—	—

* No. 3, full diet. The animals were killed one hour after feeding. No. 4, six hours after feeding.

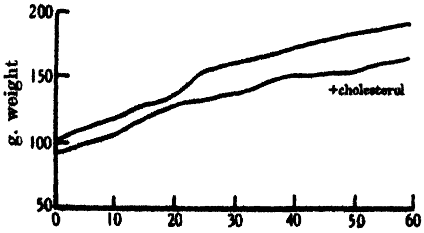

Fig. 3.

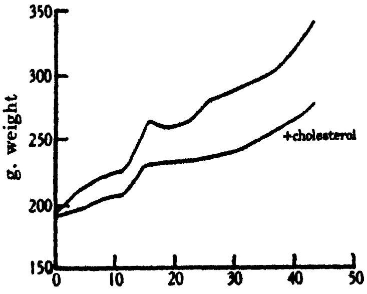

Fig. 4.

Fig. 3. Growth curves of rats fed on the laboratory normal stock diet alone and plus 2 % cholesterol. The weights are the average of four females in each series.

Fig. 4. Growth curve of guinea-pigs fed on stock diet and on stock diet plus 2 % cholesterol. The weights are the average of two animals in each set.

action on the depot stores of fat estimated as carcass fat is noteworthy. An interesting observation with the animals fed on the fat-free diet is the low fat content of the liver in the earlier periods of growth and this notwithstanding the fact that the depot stores of fat are high. In general it may be said that livers with a fat percentage greater than 5 % show the typical "fatty" appearance.

As the cholesterol effect is so marked with a fat-containing diet the possibility suggested itself that the cholesterol was only absorbed in the presence of fat. The faeces of some of the animals were therefore collected, the animals being placed in metabolism cages. For the estimation of lipins in the faeces, the latter were dried at 100° to constant weight. They were then powdered and extracted in a Soxhlet apparatus for 2–3 days with light petroleum, B.P. 50–60°. The results obtained are given in Table IV.

Table IV. *The lipin content and unsaponifiable matter in the faeces of rats fed on various synthetic diets.*

Diet and no. of rats		Days	Dry wt. g.	Total lipin g.	Unsaponifiable matter g.	Cholesterol in faeces as unsaponifiable matter in faeces minus that in control g.	Cholesterol in food over collection period g.	Cholesterol excreted %
Fat-free	2	7	7·7	0·49	0·36	—	—	—
Plus cholesterol	2	7	11·6	4·13	4·01	3·65	3·8	96
Normal synthetic	1	4	1·5	0·21	0·14	—	—	—
Plus cholesterol	1	4	2·0	0·69	0·66	0·52	0·6	87
Normal synthetic	2	7	9·0	1·54	0·34	—	—	—
Plus cholesterol	2	7	11·5	4·15	2·28	1·94	3·4	57

It will be seen from Table IV that there is practically complete excretion on a fat-free diet. The absorption is far from complete even on the fat-containing diet. Individual differences in the excretion of cholesterol in the case of the rats on this diet will be observed, and these are reflected to some extent in differences in fat in the liver. Experiments are at present in progress to study the question of absorption on diets containing varying amounts of fat.

(*b*) *Experiments with guinea-pigs.* As is well known these animals cannot be easily reared on a synthetic diet. The animals were therefore fed on a stock diet. This consisted of bran oats and egg yolk (A), and mangold roots or swedes or cabbages (B) were also given. The food intakes were on the average per animal per day 35 g. A and 40 g. B. The animals used in these experiments were about 6 months old. The cholesterol was mixed with the foodstuff A so that it was in a concentration of 2 %. Thus approximately 0·8 g. cholesterol per animal per day was eaten. The growth curves of the control animals and those given cholesterol are shown in Fig. 4.

Table V gives the results of the fat estimations on the animals.

The cholesterol feeding has a marked effect on these animals. A point of interest here is that the liver size is greatly increased. The liver of a cholesterol-fed animal may be as much as 33 % larger than of the control.

The question of the absorbability of the cholesterol was also investigated. The results are shown in Table VI.

The figure for unsaponifiable matter in the normal diet has been brought to the amount present in the same weight of faeces as in the cholesterol-fed animals in making the correction for the amount of cholesterol in the faeces.

Table V. *The fat contents of the livers and carcasses of control and cholesterol-fed guinea-pigs.*

The second animal in each set has been fed on the cholesterol-containing diet.

No.	Days on diet	Wt. of animals g.	Wt. of liver g.	Liver as % of body wt.	"Fat" in liver %	Carcass g. fat	Carcass As % of body wt.
1	40	445	16·5	3·7	2·46	20·8	4·9
		409	20·6	5·0	12·6	12·5	3·2
2	45	351	24·6	7·0	1·86	—	—
		360	36·8	10·4	6·57	—	—
3	57	367	14·7	4·0	2·92	16·9	4·9
		262	20·7	7·9	6·7	8·3	3·6

Table VI. *Cholesterol content of guinea-pig faeces.*

Two animals were on the normal diet and three on the same diet plus cholesterol. The faeces were collected over 4 days.

	Dry wt. g.	Total lipin g.	Unsaponifiable matter g.	Cholesterol in faeces as unsaponifiable matter in faeces minus that in control g.	Cholesterol in diet g.	Cholesterol excreted %
Normal diet	95	1·10	0·57	—	—	—
Plus cholesterol	113·5	6·35	4·90	4·22	8·0	53

It will be seen that there has been very definite absorption of the cholesterol in these animals and this notwithstanding the fact that the diet does not contain fat in large amounts as does the rats' diet.

DISCUSSION.

This communication must be regarded as preliminary in nature. The purpose aimed at is a study of the action of cholesterol in producing such marked disorders of fat metabolism. The growth curves show that cholesterol feeding of rats maintained on synthetic diets has only one effect and that deleterious. This action is found only when the diet contains fat. On fat-free diets there is no effect, either beneficial or otherwise, and this has been shown to be due to the absence of absorption. On the fat-containing diet the growth rate is decreased and the fatty liver develops. On a normal stock diet cholesterol is without action. There appears to be an influence of some dietary factor, possibly choline [Best, Channon & co-workers], in the difference observed between normal dietary caseinogen and caseinogen that has been extracted to remove ether-soluble matter. Thus over a period of 60 days while the control animals in both series A and B gained 156 g. in weight those given cholesterol in series A gained 139 g., those in series B only gained 100 g. With series A there was no significant difference in the food intakes of controls and cholesterol-fed animals. A similar result was found for the females on the same diet. In both series however fatty livers were found. As no experiments were made on the absorption of cholesterol in the series A animals or in those given the stock diet it is impossible to state how much of this action is due to the choline effect. In studies with cholesterol it is perhaps pertinent to include a study of cholesterol excretion. In the case of the rat the action of the cholesterol appears to depend definitely on the absorption taking place in the presence of free fat in the diet. Thus on transferring a rat which had been reared on the stock diet to the normal synthetic diet plus cholesterol the typical fatty liver developed. With regard to the question of absorbability it is of interest that the guinea-pig is able to absorb relatively large amounts of cholesterol and this on a diet which contains little fat. The experiments of Page & Menschick [1932] on another herbivore, the rabbit, show that as much as 69 % of the ingested cholesterol was either destroyed or (to a small extent) stored in the animal.

Some of the organs were subjected to a histological examination. I am indebted to Dr T. Day of the Pathology Department for his help. With the rat livers although the liver cells were full of fat there did not appear to be any marked degenerative or necrotic changes. In the case of the rats the relatively small increase in liver weight as compared with that in the controls is to be remarked. Examination of the aorta revealed little evidence of atheroma, although there were some signs in animals which had been fed for an extended period. On the other hand in the guinea-pigs' livers there was evidence of degenerative changes. The liver weight was increased considerably over that of the controls. Sections of the liver showed marked degeneration and necrosis, principally central. Sections of the aorta showed atheromatous changes. A more extended study of the pathological changes is in progress with Dr G. P. McCullagh.

In comparing the rat and the guinea-pig the species difference and difference in diet are to be considered. In general it may be said, however, that the changes induced by cholesterol feeding are of the same nature, being different only in degree.

SUMMARY.

1. The growth of rats and guinea-pigs fed on various diets containing cholesterol has been investigated.

2. Cholesterol affects the growth curve of rats on a synthetic diet only when fat is present.

3. Guinea-pigs are very susceptible to the action of cholesterol in their stock diet, which contains but little fat.

4. Cholesterol is absorbed by rats only when fat is present in the diet.

5. Fatty livers are produced in the rats on the fat-containing diet only, but in the guinea-pigs on the stock diet also.

6. There is a marked decrease in the depot fats. The action of the cholesterol appears in part to be in mobilizing the fat in the liver.

It is with much gratitude that the writer thanks Sir F. G. Hopkins for his very great kindness and the hospitality of his laboratory. He is also indebted to the Medical Research Council for a personal grant. The animals were in the competent hands of Miss V. R. Leader to whom his thanks are due.

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CCXXXII. RESPIRATION AND KETOGENESIS IN THE "CHOLESTEROL" FATTY LIVER.

BY ROBERT PERCIVAL COOK AND NORMAN LOWTHER EDSON.[1]

From the Biochemical Laboratory, Cambridge.

(*Received 18 July 1936.*)

THE respiration of tissue slices cut from normal and fatty livers has been investigated by Welch *et al.* [1935], who found that oxygen consumption decreased regularly with increasing fat content. The fatty livers were produced by feeding on a diet low in choline; and these workers were concerned with the effect of choline on the oxygen consumption. Best *et al.* [1936] consider that the diminished oxygen uptake cannot be attributed wholly to dilution of active protoplasm with fat.

We have studied respiration and ketogenesis in the livers of a small number of rats and guinea-pigs which were fed on various diets adequate with respect to choline, but containing 2 % of cholesterol. Since some of the livers showed extensive fatty infiltration, it was thought that this change would be reflected in the respiratory and fatty acid metabolism.

METHODS.

The composition of the diets, the methods of fat estimation, the effects on growth and the pathological appearances are described in the preceding paper [Cook, 1936]. Respiration and ketone-body formation were investigated by means of the tissue slice technique.

Conical Warburg cups with one side-bulb were used. The liver slices (about 20 mg. dry weight) were immersed in 3·0 ml. phosphate saline [Krebs, 1933], *p*H 7·4, and shaken under an oxygen atmosphere for 2 hours at 37·5°, during which the oxygen consumption was measured. After removal of the slices and the caustic alkali acetoacetic (β-ketonic) acid was determined manometrically by the aniline citrate method [Edson, 1935].

The metabolism is expressed by the usual quotients, Q_{O_2} and Q_{Acac}, calculated from dry weights of tissue.

EXPERIMENTAL RESULTS.

The data concerning the animals, the diets, liver weights and percentages of ether-extractable lipins (liver "fat") are given in Table I. Respiration and ketogenesis of control and of cholesterol-fed animals were examined both in the absence of added substrate and in the presence of sodium butyrate, the initial concentration of which was 0·01 *M* (Table II). The serial numbers of experiments in Table II correspond to those in Table I.

DISCUSSION.

The results show the following facts.

1. Rats which received the stock diet (Exp. 1) and the fat-free diet failed to develop fatty infiltration in spite of cholesterol feeding. The livers of these animals gave a normal respiration in absence of added substrate and the usual increase

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[1] Beit Memorial Research Fellow.

Table I. *Liver changes in rats and guinea-pigs fed on diets containing 2 % of cholesterol.*

No. of exp.	Diet	Days on diet	Wt. of animal g.	Sex	Wt. of liver g.	Naked-eye appearance of liver
		A. Rats.				
1	Stock diet	20	378	♂	17·6	Normal
	Stock diet + cholesterol		382		18·3	,,
2	Fat-free	131	284	♂	12	Normal
	Fat-free + cholesterol		265		11·3	,,
3	Normal synthetic	41	190	♂	9·7	Normal
	Normal synthetic + cholesterol		132		7·8	Fatty
4	Normal synthetic	107	302	♀	12·4	Normal
	Normal synthetic + cholesterol		354		12·8	Fatty
5	Normal synthetic	117	274	♂	11·5	Normal
	Normal synthetic + cholesterol		237		12·6	Fatty
		B. Guinea-pigs.				
6	Stock diet + cholesterol	15	265	♂	13	Normal
7	Stock diet	40	445	♂	16·5	Normal
	Stock diet + cholesterol		409		20·6	Fatty
8	Stock diet	57	367	♂	14·7	Normal
	Stock diet + cholesterol		262		20·7	Fatty

Table II. *Respiration and ketogenesis in liver slices of the same animals.*

		Controls			Cholesterol-fed animals		
No. of exp.	Substrate	Q_{O_2}	Q_{Acac}	Liver "fat" %	Q_{O_2}	Q_{Acac}	Liver "fat" %
			A. Rats.				
1	Nil	−10·1	0·97	2·50	−10·2	0·77	2·63
	Butyrate	14·9	4·88	—	−14·8	3·65	—
2	(*a*) Nil	−12·3	0·23	1·41	−13·7	0·57	1·33
	Butyrate	−16·3	2·13	—	−16·4	2·75	—
	(*b*) Nil	−14·2	0·46	0·23	−13·2	0·44	0·54
	Butyrate	−17·5	3·82	—	−16·2	2·62	—
3	Nil	−12·3	1·23	4·43	12·4	1·01	8·25
	Butyrate	−14·3	4·54	—	−13·2	4·72	—
	Glucose, 0·01 *M*	−12·9	1·26	—	−12·3	0·98	—
4	Nil	−12·5	0·87	1·11	−9·5	1·07	12·5
	Butyrate	−14·8	3·01	—	−10·6	2·89	—
5	Nil	−13·4	0·72	3·6	−9·4	1·31	12·7
	Butyrate	−15·5	3·93	—	−10·7	3·18	—
			B. Guinea-pigs.				
6	Nil	—	—	—	−6·4	0·46	2·39
		—	—	—	−6·8	0·43	—
	Butyrate	—	—	—	−9·6	2·21	—
		—	—	—	−9·8	2·12	—
7	Nil	−5·6	0·08	2·46	−5·3	0·47	12·6
		−5·8	0·11	—	−5·5	0·47	—
	Butyrate	−9·8	2·48	—	−8·8	1·95	—
		−9·6	2·26	—	−8·5	1·78	—
8	Nil	−7·1	0·49	2·92	−7·0	0·07	6·70
	Butyrate	−10·1	1·96	—	−9·9	1·03	—

of oxygen uptake and ketone-body formation in presence of butyrate. The guinea-pigs which were killed after 15 days on the stock diet plus cholesterol also presented a normal metabolism (Exp. 6).

2. Guinea-pigs kept on the cholesterol-containing diet for a longer period exhibited no significant change in respiration, but the ketone-body formation in presence of butyrate was less than that of the controls (Exps. 7 and 8).

3. The liver tissue of rats fed on the normal synthetic diet plus cholesterol for a long period (Exps. 4 and 5) had a significantly lower respiration than the controls both in presence and absence of added butyrate. The values of Q_{Acac} show no significant differences except in Exp. 5. In Exp. 3 where the time was shorter and the fat deposition less the diminished oxygen uptake was not observed.

4. The "spontaneous" ketogenesis (i.e. in absence of added fatty acid) found in the cholesterol fatty liver is not of such a high order as that which occurs in livers of rats starved 24 hours. In such animals the value of Q_{Acac} is 2 to 2·5 or even higher.

The metabolic quotients were recalculated on the basis of "fat-free" dry weights, but the figures were not sufficiently numerous to permit an analysis that would prove or disprove the view that the lowering of respiration is caused by dilution of active tissue with fat. In general it may be stated, however, that there are no great changes in respiration or ketone-body formation in the fatty livers which we have examined.

One of us (R. P. C.) is indebted to the Medical Research Council for a personal grant. Our thanks are due to Sir F. G. Hopkins for his encouragement.

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CCXXXIII. ISOLATION OF ACETIC ACID FROM MAMMALIAN LIVER.

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(*Received 18 July 1936.*)

In view of the generally implied importance of acetic acid as a degradation or fission product in intermediary metabolism, it is remarkable that no significant quantities of this substance have been found in mammalian tissues. The work which is described in this paper arose from an investigation of the alcohol-soluble constituents of ox liver, in the course of which 2:4-dinitrophenylhydrazine was added to the suitably concentrated extract in the hope of isolating carbonyl compounds of importance in intermediary metabolism. In addition to finding as a hydrazine derivative one compound which evidently possesses carbonyl groups, acetic acid was noticed in the form of its 2:4-dinitrophenylhydrazide. The results are also of interest as much for what was not discovered as for what was: acetaldehyde, pyruvic acid and kindred compounds appeared to be totally absent, although the experiments were done in the presence of sodium bisulphite (the "Abfang" method of Neuberg). The use of bisulphite has been successful in the investigation of muscle metabolites [Case & Cook, 1931] and there is no reason to suppose that intermediates containing the CO grouping would fail to be detected in mammalian liver using the same technique.

Experimental.

Isolation of hydrazide. About 4 kg. (10 lb.) of ox liver obtained from the slaughter-house less than an hour after killing were minced into a solution of 100 g. sodium sulphite (dissolved in 250 ml. water and saturated with sulphur dioxide). The mass was ground with sand (1 kg.), allowed to stand 15 min., and the protein removed by precipitation with 2 litres of 97 % alcohol. The thick paste was squeezed as free from liquid as possible by means of a hand screw-press and the extraction repeated with a further litre of alcohol. The united filtrates were kept at 0° overnight, centrifuged from protein, filtered through kieselguhr and evaporated down to small bulk (about 500 ml.) *in vacuo* at 30–40°. A certain amount of protein separated and was removed by filtration. A slight excess of conc. HCl was then added and most of the SO_2 blown off in a current of air. The solution was adjusted with HCl to make the solution approximately $2N$, and 30 g. of 2:4-dinitrophenylhydrazine dissolved in 20 ml. conc. HCl were added. After a further passage of air through the solution it was allowed to stand for 24 hours at 37°.

The precipitate, which included some unchanged reagent, was centrifuged off, boiled several times with 10 % alcohol and filtered. The residue was reserved for the osazone preparation. The cooled united filtrates were extracted 4–5 times with 50 ml. lots of ethyl acetate. After drying as far as possible over anhydrous sodium sulphate, the extract was evaporated *in vacuo* to small bulk and set aside to crystallize. In 2–3 days orange-yellow crusts separated. After draining from the dark red mother-liquor, the semicrystalline mass was dissolved in boiling

10% alcohol, a little blood charcoal added, filtered and allowed to cool; yellow plates, which were sometimes tinged a pale reddish brown, separated out. Recrystallization was effected from 10% alcohol containing a trace of HCl; M.P. 195–196° (uncorr.). Analysis: found (Weiler) C, 37·39, H, 3·91; N, 22·07%. $C_8H_{10}O_6N_4$ requires C, 37·19; H, 3·90; N, 21·71%. Dissolved in pyridine-ethyl acetate the substance showed no optical activity. Attempts to determine the molecular weight in nitrobenzene or glacial acetic acid gave anomalous results, which suggested that the substance was hydrated or contained water of crystallisation. Drying at 100° *in vacuo* over phosphorus pentoxide confirmed this. Loss in weight found 7·08% (Weiler), 7·10%; $C_8H_8O_5N_4$, H_2O requires 7·01%. Analysis of anhydrous substance: found (Weiler) C, 40·24, 40·20; H, 2·89, 3·09; N, 23·31%. $C_8H_8O_5N_4$ requires C, 39·98; H, 3·36; N, 23·34%. Mol. wt. (cryoscopic) in dry nitrobenzene; found 252; calc. 240.

The compound is evidently the 2:4-dinitrophenylhydrazide of acetic acid [Curtius & Dedichen, 1894; Purgotti, 1894]. The melting-points given in the literature vary from 193 to 197°. A specimen prepared by refluxing reagent and glacial acetic acid for one hour, pouring into water and recrystallizing, melted at 195°; mixed with the substance from liver, 195–196°. In crystalline forms and solubilities the compounds from both sources are identical. The compound gives a red colour with alcoholic potash. It is soluble in a solution of sodium carbonate and in this resembles the acid hydrazones. Unlike them, however, it cannot be titrated with alkali [Clift & Cook, 1932, 2]. The hydrazide is fairly easily hydrolysed and care must be taken that it is not heated in the presence of excess acid.

Several criticisms may be levelled against the procedure. (*a*) The acetic acid may arise from the ethyl acetate. This possibility is unlikely for several reasons: (i) If ethyl acetate be used to extract the reagent from its solution in 2*N* HCl and then the extract be dried and allowed to evaporate spontaneously in the usual way, the crystals obtained are those of the unchanged reagent, uncontaminated with hydrazide. Many repetitions of this experiment have given negative results. (ii) The Neuberg-Case method for the estimation of pyruvic acid in muscle employs ethyl acetate for extraction: yet no appreciable quantities of acetic acid are formed as an artefact. (iii) The hydrazide is soluble in sodium carbonate, yielding a deep red coloured solution. This reaction may be employed to isolate the compound without the intervention of any organic solvent whatever. Incidentally this fact and the deep red colour obtained with alcoholic potash throw considerable doubt on the validity of the Neuberg-Case method for the specific estimation of pyruvic acid. The red colour may as well be due to acid hydrazides as to acid hydrazones and is therefore no proof of the presence of carbonyl compounds.

Alternatively (*b*) the acetate radical might have been derived from the alcohol used or from impurities in the sulphite. Control experiments were performed using purified sodium bisulphite (from pure sodium carbonate saturated with SO_2). The ethyl alcohol was refluxed for some hours with barium hydroxide and redistilled. For extraction, butyl alcohol redistilled from KOH was used. Under these conditions the same hydrazide was formed and it is difficult therefore to resist the conclusion that acetic acid occurs in some quantity in the liver. The amount depends on the speed with which it can be treated with bisulphite. Under favourable conditions 4 kg. of liver yield about 16 g. of hydrazide, or 4 g. of acetic acid, which is therefore present to the extent of nearly 0·1% of the wet weight.

Isolation of the osazone. The solid residue from the hydrazide preparation, which still contains unchanged reagent, is boiled several times with 2*N* HCl, washed well, dissolved in pyridine, filtered and reprecipitated by acidification with HCl. The process is repeated and the brick-red osazone finally recrystallized from 97 % alcohol (from which it separates in microcrystalline form): M.P. 175° (decomp.) Analysis: found (Weiler) C, 42·32; H, 3·58; N, 20·12 %. $C_{20}H_{20}O_{12}N_8$ requires C, 42·5; H, 3·54; N, 19·85 %. Mol. wt. in nitrobenzene: found 482; calc. 564.

It may be remarked that the determination of the molecular weight of this compound is not facilitated by the fact that its solubility in the ordinary organic solvents is very slight. We therefore should like to make a reservation as to any definite statement on the exact molecular weight. The compound appears to the osazone of a substance of empirical formula $C_8H_{12}O_6$. It gives a blue colour with alcoholic potash. It is worth remarking that the osazone has the same empirical formula as that of the hydrazone of acetoacetic acid [Clift & Cook, 1932, 2].

A peculiar fact was noticed during the preparation of the concentrated liver extract. The bisulphite-binding power was estimated by the method of Clift and Cook [1932, 1] and it was found that the titration invariably doubled on concentration. Whether this is due to a reversal of enolisation or to a change involving two molecules has not yet been decided. The bisulphite-binding power is largely destroyed on heating the solution with an excess of alkali. The table gives the iodine equivalent of bisulphite-binding substances in various batches of liver extract.

Table 1.

No.	Wet weight of liver g.	ml. of $N/10$ iodine		Calculated figures for 100 g. liver as ml. of $N/10$ iodine for bisulphite-binding compounds before concentration
		Before concentration	After concentration	
1	2300	735	1200	32
2	4000	1840	3960	46
3	2300	783	1585	34
4	280	133	270	47·5

DISCUSSION.

The most obvious source of the acetic acid is from higher fatty acids either by splitting of the chain or by successive β-oxidations. In the absence of definite observations, however, we are not prepared to hold this view to the exclusion of other possibilities. The absence of the simpler carbonyl compounds is remarkable for these have frequently been postulated in schemes for the intermediary metabolism of fats and carbohydrates. The bisulphite-binding power is almost wholly, if not entirely, accounted for by the compound which has been isolated as an osazone and which appears to contain 8 carbon atoms. This is not improbably a condensation product: attempts are being made to determine its constitution and the mode of origin. Glucosazone has not been found under the experimental conditions employed.

SUMMARY.

1. Acetic acid, as its 2:4-dinitrophenylhydrazide, has been isolated from fresh ox liver.

2. The osazone of a compound apparently containing 8 carbon atoms has been shown to represent the major, if not the entire, part of the bisulphite-binding compounds of liver extract.

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CCXXXIV. THE TRANSMISSION OF VITAMIN A FROM PARENTS TO YOUNG IN MAMMALS.

V. THE VITAMIN A AND CAROTENOID CONTENTS OF HUMAN COLOSTRUM AND MILK.

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FEW data have been recorded on the vitamin A content of human colostrum and milk. Kennedy *et al.* [1923] reported that 10 ml. of milk from women on an adequate diet were a sufficient daily dose for the rat. Macy *et al.* [1927] have shown that 2·5–3 ml. daily of pooled milk from wet-nurses in Detroit provided sufficient vitamin A for growth and reproduction in the rat; and further, in 3 women on an abundant diet studied individually, the milk had a similar vitamin A potency, which could not be increased by supplementing the diet with 15 ml. of cod liver oil daily [McCosh *et al.* 1934]. Debré & Busson [1933] prepared from several samples of human milk the unsaponifiable matter containing the vitamin A and carotene and fed this material to rats on a vitamin A-deficient diet. They found that the unsaponifiable matter from 3 ml. of human milk was not a sufficient daily dose to keep the rats alive, but double this amount supported life and promoted slow growth. None of these workers, however, employed a biological test in which the growth-promoting activity of the human milk was compared directly with that of a standard source; and in view of the results of Coward *et al.* [1933] only a test involving direct comparison with a standard can be regarded as yielding quantitative data. Van Eekelen & de Haas [1934] applied the Carr-Price test for vitamin A and the direct colorimetric test for carotene to the colostrum and early milk of 4 women and to the later milk of 3 other women and concluded that "colostrum contains more carotene and vitamin A than milk excreted later on"; also that the later milk contained about as much carotene and vitamin A as cow's milk. They give no indication of the method by which the samples were extracted, which is a technical point of the first importance, and examination of their protocols shows that large variations of vitamin A content of samples taken from the same women on successive days were found, suggesting a lack of uniformity in the method of collecting the samples. The importance of avoiding this is dealt with below. Menken has published [1934, 1] an average figure obtained by him for the vitamin A and carotenoid contents of 49 samples of human milk; but elsewhere [1934, 2] the same figure is stated to be the average of measurements of colostrum.

In an earlier study the writer has shown [Dann, 1933] that the colostrum of the cow may be as much as one hundred times richer in vitamin A than the later milk of the same animal, and that the colostrum is therefore of considerable value in giving the calf at birth a reserve supply of vitamin A. On the hypothesis of the complementary functions of the placenta and colostrum in supplying certain materials to the foetus and new born [Needham, 1931], it would be expected that in the human subject with a more highly developed placental barrier than the cow, the colostrum would play a less important part in providing a vitamin A reserve for the infant.

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In order to test this prediction quantitative data are required for the vitamin A contents of the colostrum and milk of a series of women; in this paper such a body of data is presented.

EXPERIMENTAL.

The collection of colostrum and milk samples. The samples examined were all collected from patients in the obstetrical wards of Duke Hospital, Durham, North Carolina. The patients included both white and negro women, from both town and country districts, but all alike were poor and subsisted on the poor diet common to the poor classes in the Southern states. No control of their diet before entering the hospital was possible, except that those patients seen in the pre-natal clinic were advised to take cod liver oil, tomato juice, iron and calcium during pregnancy. In the hospital the patients received a varied and liberal diet arranged by the dietist.

The procedure adopted in collecting the samples of colostrum and milk was arranged so as to avoid as far as possible the influence of known disturbing factors on the concentration of the various constituents. Widdows & Lowenfeld [1933] have shown that the fat content of a sample of milk from a given patient is dependent upon the pressure exerted on the areola and nipple during extraction and inversely dependent upon the quantity of milk in the breast at the time the sample is taken, precisely as is found in milking cattle. Although evidence will be presented below to show that the secretion of vitamin A into the milk does not proceed parallel with the secretion of fat, the vitamin A in the milk is undoubtedly dissolved in or linked to the fat, and it is necessary to adopt a uniform method of extracting samples which will control the effect of pressure and of volume of milk in the breast. All samples were therefore extracted by use of the Abt mechanical breast pump, set to give a maximum negative pressure of 8 lb. per square in. An attempt was made to extract the sample of colostrum from each patient as soon after delivery as was possible, and if the flow had not commenced, attempts were made at intervals until the sample was obtained. The time after delivery until the sample of colostrum could be extracted varied from 4 to 110 hours, but the sample was always taken soon after secretion commenced. The samples of later milk were taken shortly before the patient left the hospital, and were all taken from the full breast approximately 5 hours after the infant had suckled it (this period being possible because the breasts were suckled alternately at successive 4-hourly feedings). It was almost always found to be quite easy to obtain 20 ml. of milk, or between 5 and 20 ml. of colostrum in this way.

Examination of the samples. The volume of the sample was determined and it was digested with concentrated KOH solution. The vitamin A and carotenoids were extracted and measured colorimetrically, the whole process being carried out exactly as previously described for cow's milk [Dann, 1933]. The results are expressed in yellow units (Y) of carotenoids and blue units (B) of vitamin A, calculated by the method of Moore [1929]. As the yellow value of pure β-carotene on this scale is about 2000 Y per mg. and as 1 B of vitamin A is approximately equivalent to 0·6 international unit (I.U.) [Moore, 1936], it is possible to estimate the total vitamin A activity of the sample by using the formula ($5/6 \times$ yellow units of carotenoids $+ 3/5 \times$ blue units of vitamin A) = I.U. of vitamin A activity. The figures so obtained will be maximum values because the yellow colour measured in the tintometer is not due to pure β-carotene, but to a mixture of carotenes together with some xanthophyll. An accurate evaluation of the vitamin A potency due to the pigments in each sample could only be made by fractionating the mixture and measuring the amounts of each pigment. In the present work this could not be done owing to the small samples available, which were often completely used up in the Carr-Price test for vitamin A after the total pigment had been measured. It has been established that the pigments of the milk fat of cows generally contain about 93 % of carotene [Gillam *et al.* 1933] but human milk fat may contain a smaller proportion of carotene. Palmer [1914], on the basis of a rough inspection of two samples of human milk, suggested that about half of the pigment present was carotene. On the other hand, Van Eekelen & de Haas [1934] also examined two samples (using the Lovibond tintometer) and found that in one the proportion of xanthophyll was too small to determine, whilst in the other it was approximately one-seventh of the total pigment. Three samples examined by the writer yielded a quantity of pigment sufficient for fractionation, and in these the carotene varied from 75 % to 90 % of the total pigment. It was decided therefore that no uniform correction could be applied to the figures obtained for the carotenoid content in order to allow for the xanthophyll present.

Data recorded. For each patient studied the age and race were recorded, and the number of the birth after which the colostrum was obtained. The date and time of birth and of the collection of the samples of colostrum and milk were also recorded, so that the length of time between parturition and the collection of the samples was known. The data obtained cover 111 women from whom colostrum was taken; milk samples were also collected from all except 7 of the group. Of the group studied 42 were white and 69 were coloured (that is, full-blooded negro, mulatto, octoroon etc.).

DISCUSSION.

Before discussing the experimental results collected in Table I it is necessary to define the use of the term colostrum. Some writers [cf. Hawk & Bergeim, 1931] apply this term to the fluid secreted by a mammalian female for a period of 2 or 3 weeks after parturition, but this usage appears to be indefensible either on etymological or physiological grounds. Colostrum is defined by the Oxford English Dictionary as "the first milk secreted after parturition by a mammal". Moreover, it is known that in species such as the cow where the colostrum differs considerably from milk in constitution (e.g. in protein or vitamin A content) a rapid change in the constitution of the mammary secretion occurs during the first 3–6 days following parturition, to be followed from then until the end of the lactation period by very much more gradual changes. In the human subject there is a much smaller difference between the constitution of colostrum and of milk, but in the one case where a comparatively large difference may occur (in the protein content of the colostrum and milk of primiparae only) Lowenfeld *et al.* [1927] found that the rapid change in composition ceased for all patients examined on or before the sixth day after delivery, which was generally 2–4 days after the first secretion of colostrum. It seems therefore that from the physiological standpoint the term colostrum should be restricted either to the first fluid secreted by the mammary glands after parturition or to all the fluid secreted before the rapid change in composition ceases. It is important to note that while the udder of the cow is filled with colostrum which is suckled reflexly by the calf at birth the secretion of colostrum in the human subject does not commence until after parturition and may be further delayed for several days.

Table I.

Mean vitamin A content, carotenoid content and estimated total biological activity of all samples of colostrum and milk examined and of samples from certain groups of subjects.

	Vitamin A content. (B per 100 ml.)	Carotenoid content. (Y per 100 ml.)	Estimated total biological activity. (I.U. per 100 ml.)
Colostrum of 111 women	629	305	632
Colostrum of 42 white women	488	301	544
Colostrum of 69 coloured women	715	307	685
Milk of 104 women	410	120	346
Milk of 38 white women	415	106	337
Milk of 66 coloured women	407	128	350
Colostrum of 39 women treated with cod liver oil during pregnancy	581	—	—
Colostrum of 72 women untreated	658	—	—
Milk of 35 women treated with cod liver oil during pregnancy	340	—	—
Milk of 69 women untreated	442	—	—

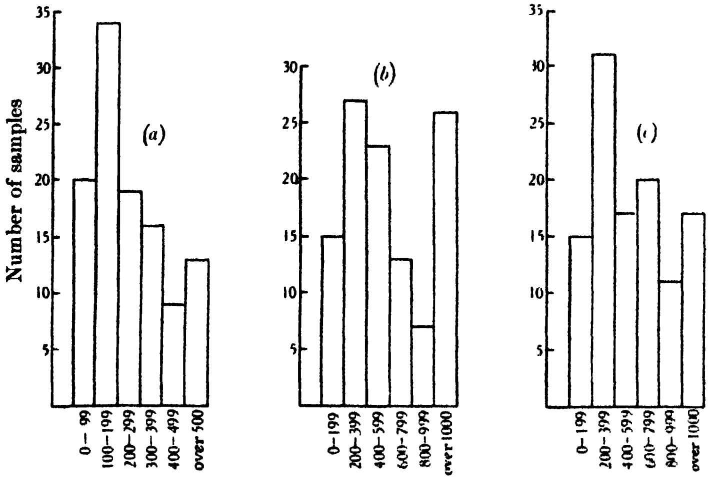
Analysis of the experimental observations. The samples of colostrum were obtained as soon as possible after parturition; that is, as soon as sufficient fluid had been secreted to allow of the collection of 5–10 ml. of colostrum. The period between parturition and the collection of the sample varied in different subjects from 4 to 110 hours. 17 samples were collected within 20 hours of parturition, 26 samples between 21 and 40 hours after, 44 samples between 41 and 60 hours after, 17 samples between 61 and 80 hours after, and 7 samples more than 80 hours after parturition. On plotting the individual figures for the vitamin A content or for the carotenoid content it was found that each of these variables was independent of the interval between parturition and the collection of the sample.

The samples of milk were collected from each subject on the day previous to her departure from the hospital, at intervals ranging from 2 to 14 days after the collection of the colostrum. 15 samples were collected within 5 days after, 68 samples from 6 to 10 days after, and 21 samples more than 10 days after the colostrum. On plotting the *ratio* of the concentration of vitamin A in colostrum to the concentration in milk against the interval between the collection of the samples, no correlation was found, showing that the change in concentration of vitamin A had gone to completion before the samples of milk were collected; the same was true for the carotenoids. (It must be remembered that the change here referred to is the first rapid change in concentration concurrent with the replacement of colostrum by milk. Other more gradual changes in the concentration of these constituents may be expected to occur over the whole period of lactation.)

The age of the subjects varied from 14 to 41 years, and they ranged from primiparae to one 11-parous subject. Here again a plot of the data showed that neither of these factors could be correlated with the vitamin A or the carotenoid content of the colostrum.

In Table I are collected mean values for the vitamin A content, carotenoid content and estimated total biological activity of all samples of colostrum and milk examined, and also mean values for samples obtained from certain specified subgroups of the subjects studied. From these figures it appears that there is no significant difference between the vitamin A contents of the colostrum (or milk) of white women and of coloured women; also there is no difference between the carotenoid contents of the milk of the two groups of women, but possibly the higher vitamin A content of the colostrum of the coloured women may be significant. This must however remain doubtful until many more data are available on account of the great range of variation for individual samples from members of each group.

Since the vitamin A content and carotenoid content of human colostrum and milk both vary over such wide ranges the mean figures given in Table I are not alone sufficient to describe the experimental findings, and some description of the distribution of the values obtained is necessary. This is contained in the histograms seen in the figures. In Fig. 1 is shown the distribution of values for the vitamin A content, carotenoid content and estimated total biological activity of the 111 samples of colostrum examined. The vitamin A content varied from 0 to 2625 B per 100 ml.; 78 samples contained less than 800 B and 50 of these had between 200 and 599 B per 100 ml. The carotenoid content varied from 20 to 1540 Y; 86 samples contained less than 800 Y, and 60 of these contained between 100 and 299 Y per 100 ml. The estimated total biological activity ranged from 56 to 2172 I.U. per 100 ml.; 80 samples contained between 100 and 799 I.U. per 100 ml. and 62 of these between 100 and 299 I.U. per 100 ml. Fig. 2 gives corresponding data for the 104 samples of milk examined. Here the vitamin A content varied from 0 to 2000 B per 100 ml.; 86 samples contained less than 800 B and 60 of these contained between 100 and 399 B per 100 ml. The carotenoid content varied from 0 to 800 per 100 ml.; 95 samples contained less than 200 Y and 80 of these less than 100 Y per 100 ml. The estimated total biological activity ranged from 0 I.U. to 1860 I.U. per 100 ml.; 62 samples contained between 100 and 299 I.U. per 100 ml.



Carotenoid content (Y per 100 ml.)

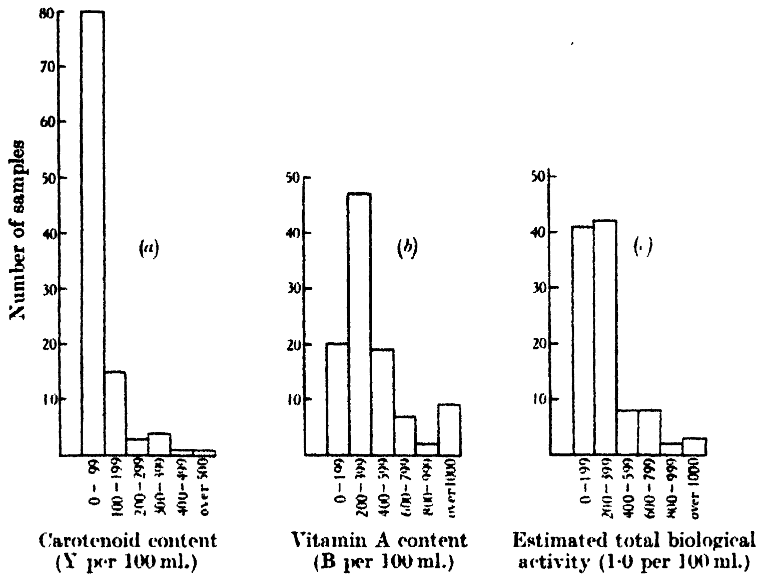
Vitamin A content (B per 100 ml.)

Estimated total biological activity (I.U. per 100 ml.)

Fig. 1. Distribution of values found for (*a*) carotenoid content of colostrum, (*b*) vitamin A content of colostrum, (*c*) estimated total biological activity of colostrum.

In Fig. 3 the distribution of values of the ratio of concentration in colostrum to concentration in milk is given for vitamin A, for carotenoids and for total biological activity. The mean values for these ratios are shown in Table II. It will be seen that the carotenoids are characteristically somewhat more specially concentrated in the colostrum as compared with the milk, than is vitamin A.

Comparison of human with cow's colostrum and milk. The data recorded here afford an interesting contrast to similar data previously obtained for the colostrum



Carotenoid content (Y per 100 ml.)

Vitamin A content (B per 100 ml.)

Estimated total biological activity (I·U per 100 ml.)

Fig. 2. Distribution of values found for (*a*) carotenoid content of milk, (*b*) vitamin A content of milk, (*c*) estimated total biological activity of milk.

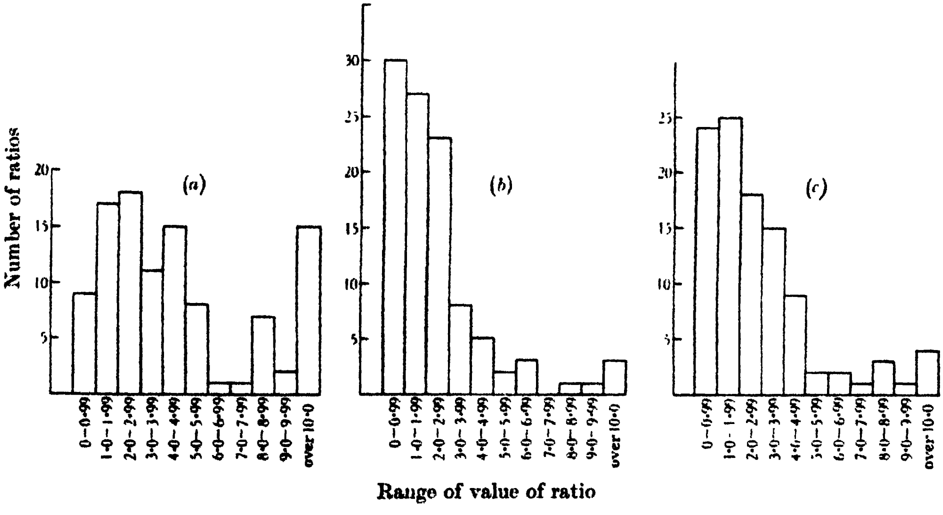


Fig. 3. Distribution of ratios found for (*a*) carotenoid content of colostrum : carotenoid content of milk, (*b*) vitamin A content of colostrum : vitamin A content of milk, (*c*) estimated total biological activity of colostrom to that of milk.

and milk of shorthorn cattle [Dann, 1933]. The mean figures for the cow's colostrum were 1253 B of vitamin A and 447 Y of carotene per 100 ml., giving a total estimated biological activity of 1055 I.U. per 100 ml. For the cow's milk the

Table II.

Mean values of the ratio of concentration in colostrum to concentration in milk for vitamin A and carotenoids, and means of the ratio of estimated total biological activities. The means are calculated to two significant figures, and two cases in which the ratio was infinity have been excluded.

Mean value of ratio for:	Vitamin A	Carotenoids	Estimated total biological activity
For all subjects (102 women)	2·4	4·8	2·7
For 37 white women	2·1	5·0	2·6
For 65 coloured women	2·5	4·6	2·7
For 35 women receiving cod liver oil during pregnancy	2·4	—	—
For 67 women untreated	2·4	—	—

corresponding figures were 30 B of vitamin A and 20 Y of carotenoids per 100 ml., giving a biological activity of 35 I.U. per 100 ml. Thus the samples of human colostrum examined in this study contained on the average about half as much vitamin A and three-quarters as much carotenoids as the cow's colostrum: the total estimated biological activity being about three-fifths as great. On the other hand the human milks were much richer than the cow's milks: they contained on the average about fourteen times as much vitamin A and about six times as much carotenoids, and their biological activity was about ten times as great. The mean value of the ratio of total activity of colostrum to that of milk was 35 for the cows examined, whilst it was 2·7 for the human subjects now studied.

Human colostrum as a source of vitamin A for the infant.

An answer can now be given to the question whether the colostrum of the human subject has any special significance as a source of vitamin A for the infant at birth, as the colostrum of the cow has for the calf. It has been shown [Dann, 1933] that the colostrum of the cow contains from 10 to 100 times as much carotenoids and vitamin A as the early milk; also that the total output of carotenoids and vitamin A on the first day following parturition may be as much as 25 times greater than the daily output in the milk 2 weeks later (unpublished observations by the writer). This is due to the favourable combination of the high concentration of vitamin A in the colostrum and the large volume of colostrum secreted (which may be as great as the daily output of milk later on).

Among the 104 women yielding both colostrum and milk samples the ratio of vitamin A activity of the colostrum to that of the milk was in general much less than the same ratio for the cow: the mean figures being 35 for the cow and 2·7 for the human subject. Also, according to Kuttner & Ratner [1923] the total secretion of colostrum by the woman is less than 100 ml., whilst the daily secretion of milk 1 week after delivery is about 500 ml. [Camerer, 1912]. In the human subject then the colostrum does not usually supply the infant with as much vitamin A as a single day's secretion of early milk, and it appears that for the human infant the colostrum has no special significance as a source of vitamin A.

It is of interest to note that colostrum so rich in carotenoids and vitamin A should be secreted by subjects receiving a poor diet during pregnancy; no figures could be obtained for subjects receiving a good diet during pregnancy, but it is hoped to extend the study to such a group in the course of further work now in progress.

SUMMARY.

1. Single samples of colostrum were obtained from 111 women shortly after secretion commenced and single samples of early milk were obtained from 104 of the women several days after the colostrum was taken. The diet of the group was uncontrolled, but poor, during pregnancy and liberal after delivery.

2. Measurements of the vitamin A content of each sample were made by the Carr-Price method, and the carotenoid content was also measured by means of the Lovibond tintometer. On the assumption that the carotenoid pigment was all β-carotene an estimate was made from these measurements of the total biological vitamin A activity in international units.

3. The vitamin A and carotenoid contents of the colostrum of women of this group are of the same order as for cow colostrum, but the vitamin A and carotenoid contents of the early milk of these women while on a liberal diet are much higher than those of cow's milk.

4. The vitamin A and carotenoid contents of the colostrum in this group could not be correlated with (a) age of patient, (b) number of birth after which colostrum was taken or (c) length of time after birth until colostrum was obtained.

5. The vitamin A content of colostrum was not increased in this group by regular ingestion of cod liver oil during pregnancy.

6. The ratio of concentration of vitamin A in human colostrum to concentration in human milk is small relative to the ratio for the cow; in approximately 70% of the cases examined it was less than 3. As the volume of colostrum in the human subject is also small, it appears that human colostrum has no special function to perform in providing a reserve supply of vitamin A for the infant at birth.

My warmest thanks are due to Prof. Bayard Carter of Duke University Medical School for much help and for permission to obtain the colostrum and milk samples from patients under his care; also to Miss M. Lewis and her staff for help in collecting the samples.

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CCXXXV. THE STRUCTURE OF UREA WITH REFERENCE TO ITS DEAMINATION AND SYNTHESIS BY UREASE.

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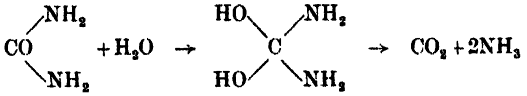
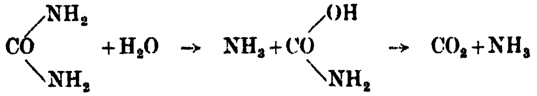
IN theory, the enzymic decomposition of urea may proceed along one of at least three possible paths:

1. Direct hydration [Armstrong & Horton, 1912],



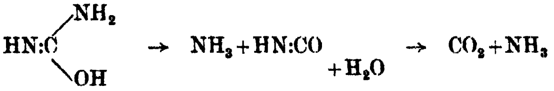
$$\mathrm{CO}\begin{matrix}\diagup \mathrm{NH_2} \\ \diagdown \mathrm{NH_2}\end{matrix} + \mathrm{H_2O} \rightarrow \begin{matrix}\mathrm{HO} \diagdown & \diagup \mathrm{NH_2} \\ \mathrm{C} & \\ \mathrm{HO} \diagup & \diagdown \mathrm{NH_2}\end{matrix} \rightarrow \mathrm{CO_2} + 2\mathrm{NH_3}$$

2. Direct hydrolysis to carbamic acid [Yamasaki, 1920; Sumner, 1926],



$$\mathrm{CO}\begin{matrix}\diagup \mathrm{NH_2} \\ \diagdown \mathrm{NH_2}\end{matrix} + \mathrm{H_2O} \rightarrow \mathrm{NH_3} + \mathrm{CO}\begin{matrix}\diagup \mathrm{OH} \\ \diagdown \mathrm{NH_2}\end{matrix} \rightarrow \mathrm{CO_2} + \mathrm{NH_3}$$

3. Dissociation into cyanic acid [Werner, 1923, 1; Fearon, 1923, 1926],



$$\mathrm{HN{:}C}\begin{matrix}\diagup \mathrm{NH_2} \\ \diagdown \mathrm{OH}\end{matrix} \rightarrow \mathrm{NH_3} + \underset{+\mathrm{H_2O}}{\mathrm{HN{:}CO}} \rightarrow \mathrm{CO_2} + \mathrm{NH_3}$$

Of these possible reactions, the first has always lacked experimental support for the existence of the hypothetical hydrated carbamide, and the work of Sumner and others has shown that the primary detectable effect of the action of urease on urea is the liberation of ammonia and another compound, now recognized to be carbamic acid. The third reaction path is the one that Werner has found to be followed in the hydrolysis and synthesis of urea by purely chemical methods, cyanic acid being an obligatory intermediate in all these changes, and it might be expected that the catalytic decomposition of urea would proceed along the same lines as those of thermal or alkaline hydrolysis.

When urea solutions undergoing zymolysis are treated with an excess of silver nitrate it is possible to obtain precipitates which resemble silver cyanate and differ from silver carbamate in their stability to acids up to pH 5; in higher concentrations of acid these precipitates are hydrolysed with liberation of NH_3 and CO_2. Furthermore, concentrated solutions of urea undergoing vigorous zymolysis were found to give, under appropriate conditions, various colour reactions characteristic of cyanates. For these reasons, the present author was led to conclude that the primary effect of urease was to catalyse the dissociation of the substrate.

Sumner *et al.* [1931], working with pure preparations of the crystallized enzyme obtained from jack bean extracts, were unable to find any evidence of cyanate formation during the early stages of zymolysis; these results have been confirmed by the present writer, using the jack bean enzyme, which necessitates the conclusion that the silver precipitates obtained in the earlier experiments were silver carbamate protected in some way by the impurities present in the original soy bean preparations. Whilst Sumner's work has revealed the importance of carbamic acid as the intermediate reactant in the zymolysis of urea, two dependent questions remain unanswered: the form in which urea is attacked by the enzyme, and the significance, if any, of the positive reactions for cyanate obtained in the later stages of hydrolysis on application of the copper-benzidine and other tests.

From the chemical standpoint, the proof that such a stable solute as urea can be deaminated by direct hydrolysis is of much interest; while from the biological standpoint, the acceptance of the highly reactive cyanic acid as a possible metabolite in the physiological history of urea has many bearings on the wider questions of the origin and fate of urea in plants and in animals.

Criticism of the carbamate theory of zymolysis.

1. The detection of carbamic acid by the method employed by Sumner does not preclude the possible existence of *iso*cyanic acid as its precursor, since carbamic acid is the first product of the hydrolysis of cyanic acid [Fearon & Dockeray, 1926].

2. Quastel [1932] has observed that potassium cyanate protects urease from the toxic action of brilliant green, whereas ammonium carbamate has little or no effect. This is evidence for combination between cyanic acid (but not carbamic acid) and the enzyme.

3. The carbamide formula for urea affords no clue as to the manner in which deamination can occur. The amino-grouping in urea is much more stable than that of the acid amides or amino-acids, and does not react with formaldehyde or with nitrous acid until it has been unmasked by the addition of a strong acid [Werner, 1923, 1].

Criticism of the cyanate theory of zymolysis.

1. The hydrolysis of metallic cyanates in solution is not accelerated by urease. When potassium cyanate is added in low concentrations to buffered solutions of boiled and unboiled urease kept at temperatures of 10–30° hydrolysis may actually proceed more slowly in presence of the active enzyme.

This difficulty was recognized and evaded in the earlier work by assuming that the cyanate liberated by dissociation of urea was in the labile *iso*-form, HN:CO, whereas the alkali cyanates are salts of more stable form, HO.CN. The existence of this isomerism has been shown from a study of the effect of temperature on the polymerization of cyanic acid [Werner & Fearon, 1920], and the recent work of Birckenbach & Kolb [1935] on the Raman spectrum of the cyanate residue has confirmed the occurrence of the two forms of the acid.

2. Cyanate cannot be detected by the very sensitive copper-pyridine test [Werner, 1923, 2], or the copper-benzidine test [Fearon, 1926], in the earlier stages of zymolysis, but only in concentrated solutions of urea which have been allowed to approximate to zymolytic equilibrium. This suggests either that the cyanate is the result of some side-reaction, or else is an artefact produced by the action of the ammonia on the enzyme.

In order to define the ambiguous position of cyanate in the urea-urease system a series of experiments was carried out in presence of reagents capable of immobilizing cyanate as well as carbamate. The conclusions reached are: (1) carbamic acid is formed by the direct deamination of urea, and not by hydrolysis of a precursor; (2) cyanic acid is formed by dehydration of ammonium carbamate, and can only appear in the later stages of the reaction when the carbamate content has reached a sufficiently high level.

Application of trap methods of analysis to the urea-urease system.

Trap methods of analysis are of two kinds: those designed to stabilize intermediate reactants as solutes, and those designed to remove intermediate reactants as precipitates. Examples of the first type employed were salts of barium and calcium, both of which form soluble cyanates and carbamates. Examples of the second type employed were (1) lead, which forms an insoluble cyanate and carbamate, and (2) the organic bases, aniline, phenylhydrazine and semicarbazide, all of which form sparingly soluble derivatives of cyanic acid but not of carbamic or carbonic acids.

The sources of enzyme employed were: (i) a high-grade sample of jack bean meal, which yielded (ii) the crystallized enzyme according to Sumner's method; (iii) a sample of soy bean meal freed from lipin material by extraction with light petroleum and with warm alcohol, and the commercial preparations of (iv) Dunning and (v) Squibb.

A. *Zymolysis of urea in presence of barium and calcium salts.*

Exp. 1. 50 ml. solutions of $M/3$ urea were treated with 1 ml. of urease solution prepared by centrifuging 1 % suspensions of (i), (iv) and (v). The mixtures were kept at 10°, and as each became alkaline to phenol red it was neutralized by the addition, every 5–10 min., of M calcium acetate in $M/40$ acetic acid. In the mixture containing urease (v) a precipitate of phosphate appeared after each addition of calcium, and was redissolved by further addition of $N/10$ acetic

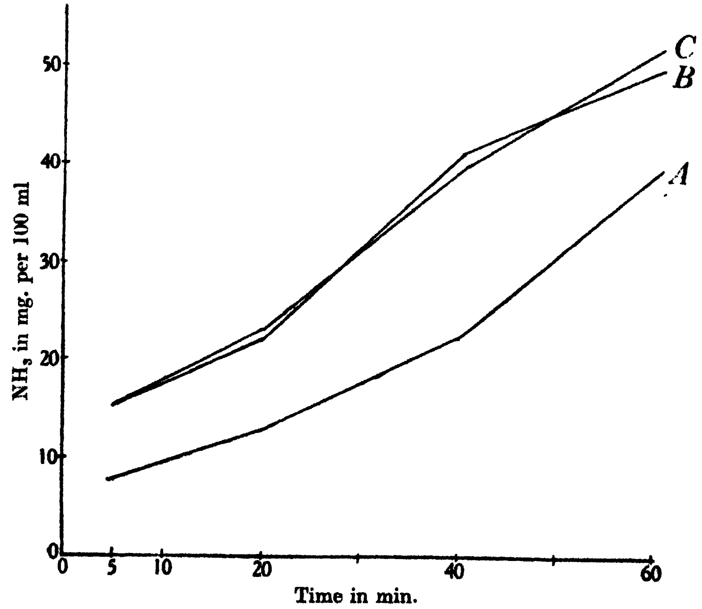


Fig. 1. Zymolysis of urea in presence of calcium acetate. *A*, "free ammonia". *B*, "free + carbamate ammonia". C, "Total ammonia".

acid. For analysis, triplicate samples were withdrawn. To the first, 1 ml. of *N* HCl was added, and the mixture kept at 40° to hydrolyze any cyanate or carbamate that might be present. Subsequent nesslerization of this sample gave the "total ammonia" value of the mixture. Each of the two remaining samples was treated with 4 ml. of *N* NaOH. The "free ammonia" in one sample was estimated by nesslerization following centrifugal separation of the precipitated calcium hydroxide. The last sample was incubated at 40° for 2 hours in order to hydrolyze any carbamate that might be present. Under these conditions, as Lewis & Burrows [1912] have shown, cyanate does not liberate ammonia. Subsequent nesslerization of this sample, after removal of precipitated calcium carbonate, gave the "free + carbamate ammonia" value of the mixture.

In all these experiments the value for the "total ammonia" was found to be the same as that for the "free + carbamate ammonia", which indicates that carbamic acid and ammonia are the only identifiable products of the primary attack of urease on urea.

In none of the experiments was any carbonate formation observed during the first hour, although ammonia was being liberated continuously. This indicates that carbon dioxide cannot be a primary product of the zymolysis of urea, as originally suggested by Armstrong & Horton, but must arise from the hydrolysis of the carbamate.

B. *Zymolysis of urea in presence of lead salts.*

Of the commoner metals available for the fixation of cyanate as an insoluble salt, namely, silver, mercury, lead and copper, only lead was found to be suitable for use with the enzyme. Now, although, as Sumner had pointed out, Pb ions, even in very low concentration, are highly toxic to pure crystallized urease, it is a remarkable fact that the crude jack bean extracts continue to attack urea even when the enzyme has been completely precipitated by lead acetate and is no longer in solution.

Exp. 2. 100 ml. of *M* urea were treated with 5 ml. of a centrifuged 5 % aqueous extract of jack bean. The mixture was kept at 10°, and as it became alkaline to phenol red it was neutralized from time to time by the careful addition of 10 % lead acetate in *N* acetic acid.

The lead produced a precipitate which carried down the enzyme, but zymolysis continued slowly after adsorption as shown by the colour change of the indicator in the region of the precipitate. After 4 hours the mixture had received 4 ml. of the lead acetate solution. The lead precipitate from half of the mixture was now collected, washed free from ammonia and urea and dried in a desiccator. The remaining half of the mixture was kept for 3 days before collecting the precipitate. The precipitates were tested for cyanate by the copper-pyridine test, and analyzed for cyanate and carbamate by the method of alkaline and acid hydrolysis. In no experiment was there any evidence of cyanate formation.

Precipitate 1. Wt., 0·41 g. Carbamate, 3·9 %.

Precipitate 2 consisted entirely of lead carbonate together with jack bean débris.

C. *Zymolysis of urea in presence of semicarbazide.*

Concurrently with the experiments on the effect of metallic stabilizers, a series was set up to see if an organic compound could be used to trap the hypothetical precursor of the carbamic acid. Aniline, phenylhydrazine and semicarbazide were the bases selected as yielding insoluble ureides with cyanic acid. In the relatively high concentrations used, *M*/10–*M*, aniline and phenylhydrazine had much greater inhibiting effects than semicarbazide, which is in agreement with the results obtained by Myrbach [1926] during his study of the action of the aldehyde reagents on yeast saccharase. In lower concentrations, Quastel has shown that semicarbazide has a protective action on urease.

Semicarbazide in acid solution reacts with cyanic acid to form a characteristic crystalline compound, hydrazodicarbonamide, $H_2N.CO.NH.NH.CO.NH_2$, the solubility of which is about 1:7000, at room temperature, and which melts (with decomposition) at 245°.

Hydrazodicarbonamide occurs in two crystalline forms, according to the conditions of the reaction. When a few drops of fresh 0·1 % potassium cyanate are added to about 2 ml. of 5 % semicarbazide hydrochloride the dicarbonamide begins to separate out in a few minutes in the form of brilliant tetrahedra. When the semicarbazide reagent is added in small quantities to a warm solution of potassium cyanate, which has been boiled for a few minutes to liberate ammonia, the dicarbonamide appears as a mixture of tetrahedra and ovoid crystals.

On recrystallization from warm water the ovoid crystals revert to the tetrahedral form. Hydrazodicarbonamide is not dissolved in the cold by *N* HCl, *N* NaOH, concentrated NH_3 or the commoner non-polar organic solvents. It dissolves with decomposition in hot, glacial acetic acid.

The semicarbazide reagent was applied to the urea-urease system in two ways. In the first set of experiments, *M* and *M*/3 solutions of urea undergoing zymolysis at 10° were neutralized from time to time by addition of *M* semicarbazide hydrochloride. When the reactions had proceeded for 3–6 hours the mixtures were acidified by addition of excess of the reagent and left overnight. Next day, and for several subsequent days, samples of each mixture were centrifuged and examined for crystal formation, but in no instance was there any sign of hydrazodicarbonamide crystals among the enzyme débris. In the second set of experiments, the urea and semicarbazide hydrochloride were mixed in equimolecular proportions, and the mixture neutralized with *N* NaOH before adding the enzyme. After zymolysis had continued for 24–48 hours at room temperature, the mixtures were acidified with *N* HCl, and placed in a desiccator. Within 24 hours a crystalline precipitate began to appear among the débris. The crystals consisted chiefly of small ovoids closely resembling the ovoid form of the dicarbonamide. Along with these crystals were a few tetrahedra, as well as the polysaccharide granules which are commonly present in suspensions of jack bean extracts. When crystallization appeared to be complete, the residue was collected in a centrifuge-tube, washed with concentrated ammonium hydroxide, *N* NaOH, *N* HCl and finally with several changes of water. The residue was then extracted three times with boiling water, the extracts collected and concentrated. On standing, typical tetrahedral crystals began to separate out, which were collected and identified as hydrazodicarbonamide by their melting-point.

Exp. 3. 2 g. of urea and 2 g. of semicarbazide hydrochloride were neutralized to phenol red by means of *N* NaOH, treated with a suspension containing 75 mg. urease (Dunning) and the total volume made up to 25 ml. After zymolysis for 24 hours the mixture was reduced in a desiccator to about 10 ml. and then slightly acidified with *N* HCl. Crystal formation was observed towards the end of the second day, and after a week the residue was collected, washed with acid and alkali and twice recrystallized from hot water. The yield of hydrazodicarbonamide amounted to 18 mg.

Cyanate formed under these conditions may have at least three possible origins: (i) decomposition of an unidentified impurity accompanying the enzyme preparation, (ii) hydrolysis of part of the semicarbazide itself and (iii) action of the urease, or possibly an associated enzyme, on one of the reactants in the mixture. The third of these possibilities is the most likely, since negative results as regards hydrazodicarbonamide formation were obtained with the following mixtures:

(1) Urease digested with semicarbazide in acid and in alkaline solutions.

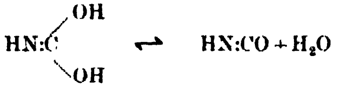
(2) Urease incubated with semicarbazide and ammonium hydroxide, chloride or sulphate.

(3) Urease incubated with semicarbazide and sodium hydrogen carbonate.

(4) Heat-inactivated urease digested with semicarbazide and urea.

(5) Heat-inactivated urease digested with semicarbazide and ammonium carbamate-carbonate.

Now although urease does not liberate cyanic acid directly from urea, the possibility remains that it, or some accompanying enzyme, is able to catalyze the dehydration of carbamic acid in solution in a manner comparable with the action of carbonic anhydrase on carbonic acid.



$$HN{:}C(OH)_2 \rightleftharpoons HN{:}CO + H_2O$$

Carbamic acid
(imino-form)

If this hypothesis be true it might be expected that when urease is incubated with concentrated solutions of ammonium carbamate-carbonate urea would be produced from the union of cyanate and ammonia; whereas if urease is incubated with the same substrate in presence of a cyanate stabilizer it should be possible to detect the intermediate reactant.

Mack & Villars [1923], and Kay [1923] were the first to demonstrate the formation of urea by the action of urease on ammonium carbamate-carbonate, thus showing that the outline reaction,

$$CON_2H_4 + 2H_2O \rightleftharpoons (NH_4)_2CO_3,$$

is capable of being carried out in either direction by the enzyme. Now, Werner has obtained a considerable amount of evidence that all the familiar syntheses of urea from ammonium carbonate by purely chemical means necessitate (i) the formation of carbamic acid, and (ii) its dehydration to cyanic acid, which then combines with the ammonia to form urea.

Consequently, it is of interest to determine if the enzymic synthesis of urea retraces the path of deamination in the reverse direction, or if cyanate formation represents an alternative route of synthesis. This aspect of the problem has a bearing on the origin of urea in lower plants, many of which appear to be free from urease [Fosse, 1916; 1928]. The possibility of course remains that cyanate formation is due to the activity of a separate enzyme, quite distinct from urease, and present in soy and jack bean extracts that have not been fractionated by Sumner's method of crystallization.

Before following this line of investigation, which will be made the subject of a later research, it is necessary to determine the optimal conditions for the enzymic synthesis of urea. These are exemplified in the following data obtained from a series of experiments carried out in collaboration with Prof. E. A. Werner, in which yields of urea considerably greater than those of previous investigators have been obtained.

The synthesis of urea by urease.

The enzyme preparations used were: (i) soy bean residues after extraction with fat-solvents, (ii) jack bean residues and (iii) urease Dunning. Other preparations were examined also, including several free from urease, in order to test the possibility that the urea might arise from sources other than true reversed zymolysis. The substrate was an ammonium carbamate-carbonate mixture, which on analysis represented 4·45 N carbamate and 6·4 N carbonate. In each experiment, 25 ml. of the substrate were incubated with the enzyme preparation in a closed flask for 8 hours at 55°.

Table I. *Urea formation from ammonium carbamate-carbonate at 55°.*

Enzyme preparation	Urea yield from 25 ml. substrate mg.
None	0·0
Jack bean, heat inactivated, 25 mg.	0·0
Jack bean I, 25 mg.	4·0
Jack bean I, 75 mg.	6·6
Jack bean II, 25 mg.	16·2
Jack bean II, 500 mg.	20·3
Soy bean, 250 mg.	1·7
Soy bean, 250 mg.	1·6
Soy bean, 750 mg.	3·2
Soy bean, lipin-free, 25 mg.	3·7
Soy bean embryo, dried, 250 mg.	1·8
Soy bean embryo, lipin-free, 250 mg.	3·0

The preparation "Jack bean II" is the urease powder marketed by Messrs Dunning.

Table II. *Incubation of preparations free from urease.*

Preparation	Urea yield from 25 ml. substrate mg.
Takadiastase, 250 mg.	0·0
Peptone, Merck, 250 mg.	0·0
Bran, dried, 250 mg.	0·0
Arginine hydrochloride, 33 mg.	(a trace)

Table III. *Incubation of urease preparations with various substrates.*

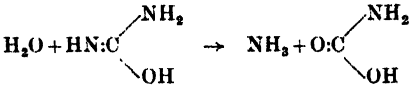
Preparation 250 mg.	Substrate	Urea yield from 25 ml. substrate mg.
Soy bean	N NaOH	0·0
Soy bean	N NH_4OH	0·0
Soy bean	2·5 N NH_4OH	0·0
Soy bean	$8N$ NH_4OH	0·0
Soy bean	$10N$ $H.COO.NH_4$	0·0
Soy bean	Carbamate-carbonate	1·6
Soy bean	Carbamate-carbonate	1·6
Soy bean	N-methylammonium-methyl carbamate	0·0

The urea formed was estimated as follows. After incubation, each mixture was concentrated to 5 ml. on a water-bath to remove excess of ammonia, then diluted with 10 ml. of glacial acetic acid, and treated with 3 ml. of 10 % xanthydrol in methyl alcohol. After remaining for 12–14 hours, the xanthydrol precipitates were removed by filtration, washed with alcohol and ether and dried and weighed, according to the technique of Fosse. The precipitates were identified as dixanthylurea from their micro-crystalline appearance and their melting-point, 260°, as taken in the vapour of diphenylether.

These experiments lead to the conclusion that the formation of urea by the action of urease on concentrated solutions of ammonium carbamate is a true enzymic synthesis and not due to the action of the strong alkali on urea precursors.

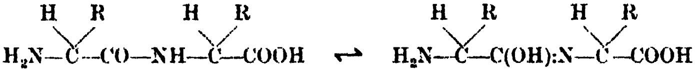
The point of attack in the action of urease on urea.

If it be conceded that the point of attack in the zymolysis of urea is the imino-group, and not the amino-group, as commonly assumed, Werner's earlier objections to the direct deamination of urea can be overcome, and, furthermore, the action of urease can be related to those of other enzymes capable of hydrolyzing the C—N linkage. That is to say, the zymolysis of urea is represented as:



$$H_2O + HN{:}C\begin{matrix}\diagup NH_2\\ \diagdown OH\end{matrix} \rightarrow NH_3 + O{:}C\begin{matrix}\diagup NH_2\\ \diagdown OH\end{matrix}$$

the polar groups, $—NH_2$ and $—OH$, serving as points of attachment for the enzyme in a manner comparable with that postulated for dipeptidase systems by Bergmann *et al.* [1935]. In general, the imino-linkage appears to be sensitive to enzyme attack when it appears in compounds containing the configuration $R—C\,(OH){:}N—R$, which occurs in many substances capable of exhibiting resonance between enol and keto forms.



$$H_2N—\overset{H\ \ R}{C}—CO—NH—\overset{H\ \ R}{C}—COOH \rightleftharpoons H_2N—\overset{H\ \ R}{C}—C(OH){:}N—\overset{H\ \ R}{C}—COOH$$

Peptide type

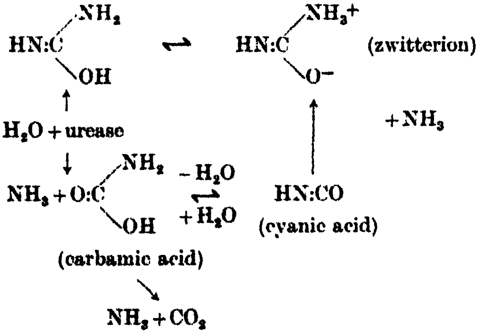
$$R—CO—NH_2 \rightleftharpoons R—C(OH){:}NH$$

Urea type

Examples of such configurations can be shown to exist at the point of attack in natural substrates such as allantoin, arginine, asparagine, guanine, hippuric acid and the peptides, and the deamination of α-amino-acids may also be explained in terms of oxidative formation and subsequent hydrolysis of an imino-group.

SUMMARY.

The following scheme is proposed as representing the changes occurring in the urea/urease system:



NOTES: ***The structure of urea.*** Of the five possible structures that may be assigned to urea, it is assumed in the above scheme that the unsymmetrical or *iso*-form is the one attacked by the enzyme. While the symmetrical or true carbamide form is found in ureides and substituted ureas, according to Werner it is too unstable to exist free in solution.

Were it present, the zymolysis of urea might be expected to follow the lines originally suggested by Armstrong & Horton, and result in the simultaneous appearance of ammonia and carbon dioxide in the solution at the start of the reaction. A cyclic or closed form of urea has been suggested by Werner to account for the fact that in neutral solution the amino group is masked and will not react with aldehydes, but this can also be explained more simply by assuming that urea is in equilibrium with a zwitterionic form, as represented above.

The unexpectedly high value obtained by Wyman [1933] for the dielectric constant of urea solutions supports the suggestion that the solute is present largely in the zwitterionic form.

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CCXXXVI. THE ACTION OF HYPOCHLORITES ON AMINO-ACIDS AND PROTEINS. THE EFFECT OF ACIDITY AND ALKALINITY.

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IN a previous paper [Wright, 1926] the author recorded the results obtained in a study of the rate of reaction between solutions of sodium hypochlorite and certain amino-acids and proteins. It was suggested that the rate of utilisation of the available chlorine was governed by at least two factors: (1) the possibility of the hypochlorite acting either as an oxidizing or as a chlorinating agent, and (2) the variable stability of the chloroamino-derivatives formed by the latter reaction. Brief reference was made in the paper to the very marked effect of alterations in the acidity of the solutions, alterations which changed completely the type of reaction curve obtained. Subsequently a number of experiments were carried out to determine more exactly the effect of varying pH values on the rate of reaction. The results of this additional work were not, however, published.

Quite recently Norman [1936] has described a detailed study of the action of sodium hypochlorite on glycine. As a result of her work she expresses doubt as to whether chlorination does, in fact, take place as an intermediate step in the oxidation of glycine by hypochlorite. Since the additional work carried out by the present author has a definite bearing on this question it has now been felt desirable to publish the results, although these are in an admittedly incomplete form.

Technique.

The experiments were carried out as described in the previous paper, except that the acidity or alkalinity of each system was controlled by the use of strongly buffered salt solutions. The solutions employed were:

for pH 1·0 to 2·0, HCl—KCl mixtures;
„ pH 5·0 to 8·0, KH_2PO_4—NaOH mixtures;
„ pH 8·5 to 10·0, borate—NaOH mixtures;
„ pH 12·5, $N/10$ NaOH solution.

The reactions were carried out for 5-hour periods, the percentage of available chlorine remaining at the end of this time being determined by liberation of iodine from an acid solution of KI and back-titration with thiosulphate. The initial strength of the available chlorine was 0·014 % in all experiments.

Experiments with glycine and cystine.

Glycine. Experiments were carried out with glycine over a wide pH range. It will be seen from Figs. 1, 2 and 3 that on either side of pH 8·8 the solutions showed more or less uniform gradations in the extent to which they reduced the available chlorine. All the solutions, with the exception of those at extreme pH values (i.e. 1·0–2·0 and 12·5), gave with low concentrations of glycine a typical straight line fall towards zero, and with higher concentrations a typical straight

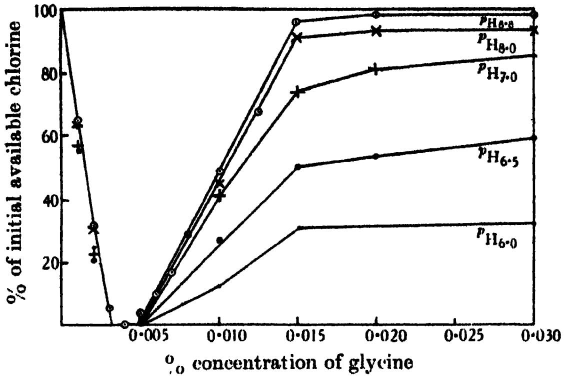

Fig. 1. Effect of glycine in reducing the available chlorine. *p*H range 6·0–8·8 (initial available chlorine 0·014 %).

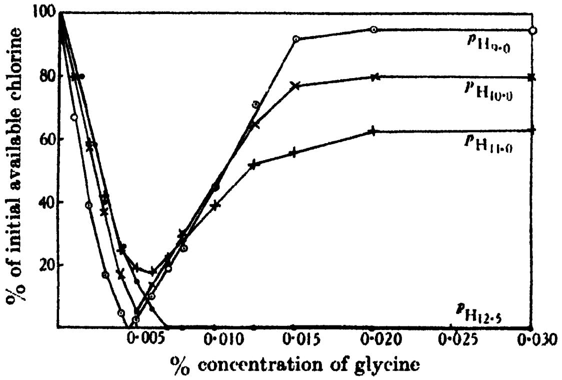

Fig. 2. Effect of glycine in reducing the available chlorine. *p*H range 9·0–12·5 (initial available chlorine 0·014 %).

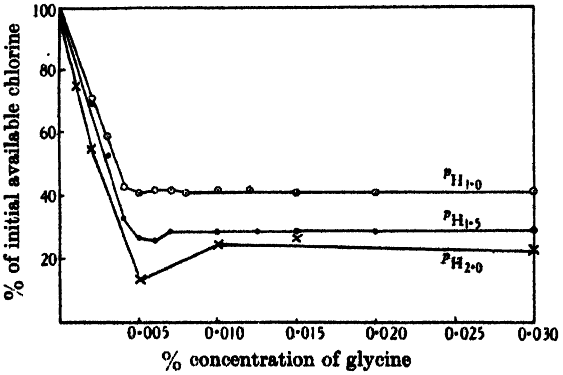

Fig. 3. Effect of glycine in reducing the available chlorine. *p*H range 1·0–2·0 (initial available chlorine 0·014 %).

line rise towards 100 %. This confirms the previous work. It will, however, be noted that the slope of the initial fall is steepest with the more acid solutions. That is to say it requires less glycine to use up a given quantity of available chlorine in the acid solutions than in the more alkaline solutions. The whole of the available chlorine in the solution is, for example, removed by less than 0·003 % glycine at *p*H between 6·0 and 8·8, whilst at *p*H 12·5 double this quantity of glycine is required. The Cl/glycine ratios at these extremes are 4·6 and 2·3 respectively. This difference can be most readily explained on the assumption that in acid solution the glycine is first chlorinated and that the resulting chloroamino-acid is then oxidized by further quantities of hypochlorite, whilst in alkaline solution the destruction of available chlorine is due to oxidation alone.

Turning to the secondary rise, it will be seen that at *p*H 8·8 there is no appreciable reduction in available chlorine when the quantity of glycine present exceeds 0·015 %. In the previous paper this fact was explained as being due to the quantitative formation of the relatively stable chloroaminoacetic acid which would be capable of liberating iodine from KI in the same quantities as the hypochlorite initially present. If this explanation is correct, the lower levels attained at *p*H on either side of 8·8 must be due to a loss of stability in the more acid and the more alkaline solutions. At *p*H 12·5, where presumably no chlorination takes place, there is of course no secondary rise.

The curves for *p*H 1·0–1·5 (Fig. 3) show entirely different characteristics from the remaining curves on the acid side of *p*H 8·8. There is no preliminary fall to zero and no secondary rise, but the curves fall to a constant value at between 25 and 40 % of the initial available chlorine. At *p*H 2·0 the type of curve is intermediate. It will be shown later that similar anomalous curves are given in extremely acid solutions by both caseinogen and gelatin.

Cystine. Only three *p*H values were used in studying this amino-acid (Fig. 4). At *p*H 8·8 the curve is similar to that obtained in the previous work. There is no secondary rise with this amino-acid, the chloroamino-derivative being very unstable and decomposing, with complete loss of available chlorine, within 5 hours at room temperature. The curve at *p*H 12·5 shows a steeper slope than that obtained at *p*H 8·8, indicating (as noted with glycine) the probable double nature of the reaction at the latter acidity. At *p*H 1·0 the results are again anomalous, the fall to zero taking place along two consecutive straight lines, one with a steep slope and one with a more gradual slope.

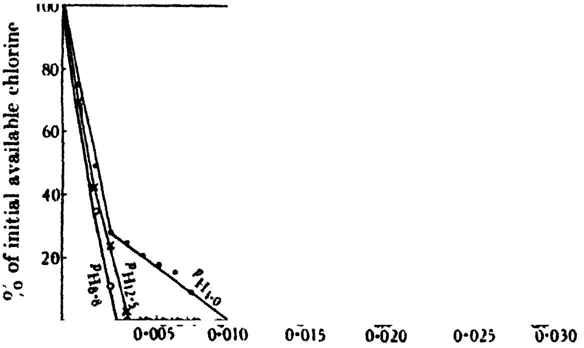

Fig. 4. Effect of cystine in reducing the available chlorine. *p*H range 1·0–12·5 (initial available chlorine 0·014 %).

With this amino-acid, one marked difference was observed between the reactions taking place in acid and alkaline solutions. With acid solutions the liquid remained colourless, except for a slight initial coloration due to liberation of chlorine in the solution. With alkaline solutions the liquid rapidly acquired a pale straw-yellow colour, the intensity of the colour depending on the concentration of cystine. This colour was found to be due to the formation of polysulphides which could be decomposed by addition of acid, with liberation of hydrogen sulphide.

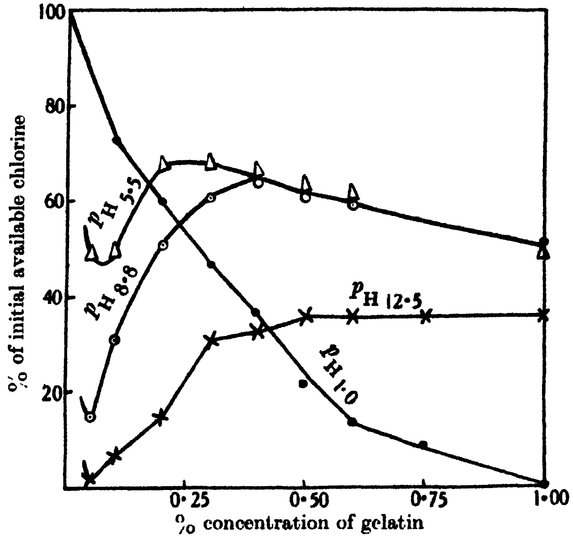

Fig. 5. Effect of gelatin in reducing the available chlorine. pH range 1·0–12·5 (initial available chlorine 0·014 %).

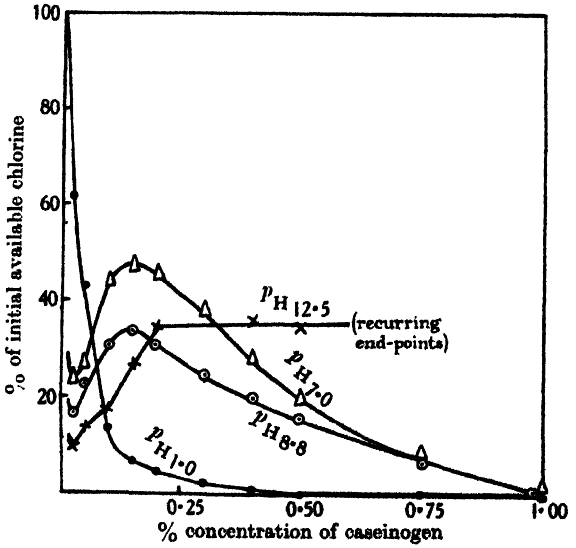

Fig. 6. Effect of caseinogen in reducing the available chlorine. pH range 1·0–12·5 (initial available chlorine 0·014 %).

Experiments with gelatin and caseinogen.

Similar experiments have been carried out with gelatin and caseinogen (Figs. 5 and 6). At *p*H between 5·0 and 9·0 the curves show a general similarity to those obtained in the earlier work, i.e. an initial fall and secondary rise, and a final but slower fall in the percentage of available chlorine. The secondary rise is smaller and the final fall is sharper with caseinogen than with gelatin, a fact which was attributed in the previous work to variations in the stability of the chloroamino-derivatives formed as a result of the hypochlorite action. It may be noted that with both proteins the highest secondary rise was obtained with *p*H on the acid side of 8·8 and not (as with the individual amino-acids) at *p*H 8·8.

The curves for *p*H 1·0 have an entirely different form from those obtained in slightly acid, neutral or alkaline solution. They show, however, a marked similarity to that obtained with a glycine-cystine mixture (Fig. 7).

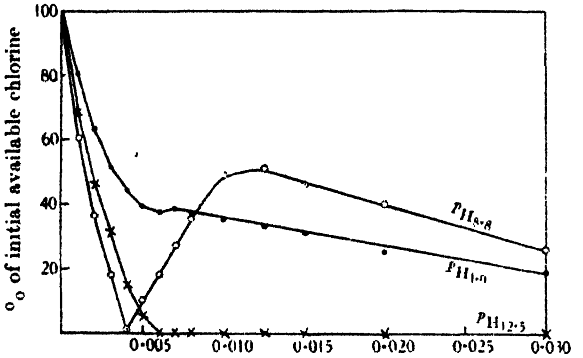

Fig. 7. Effect of a glycine/cystine mixture in reducing the available chlorine. *p*H range 1·0–12·5 (initial available chlorine 0·014 %).

At *p*H 12·5 the proteins fail to show the same type of curve as those given with either the individual or the mixed amino-acids. The curves for both proteins show an initial fall to a relatively low figure, the available chlorine being reduced to a very small fraction of its initial strength. Thereafter the curves rise to a constant level of roughly 35 %—the titrations tending to give recurring end-points. No explanation can be given for this phenomenon.

One further point may be mentioned. In carrying out a number of supplementary experiments with gelatin and caseinogen it was found that, using a ratio of protein/available chlorine of 0·050 %/0·014 % and in solutions of HCl varying between 0·1 and 0·02 *N*, a precipitate flocculated out within a few minutes. This precipitate was slightly yellow and was capable of liberating iodine from KI. It was, however, decomposed by the addition of alkali, with the formation of a light greenish yellow solution. The precipitate was not formed at other acidities and only appeared when the ratio of protein/available chlorine was approximately that quoted.

DISCUSSION.

In a recent paper Norman [1936] cites experiments which, she states, indicate that hypochlorites do not normally react with amino-acids to form chloroamino-derivatives, but that "the theory of direct oxidation to cyanide seems at least as well founded as that of the formation of chloroamino-acids". This is in direct opposition to the views expressed by the present author [Wright, 1926] that hypochlorite may act either as an oxidizing or as a chlorinating agent, the nature of the reaction depending on the relative quantities of the amino-acid and of the available chlorine present.

In her preliminary experiments with glycine Norman obtained curves which were in general similar to those obtained in the author's earlier work, discrepancies in the rate of reaction probably being due (as she suggests) to differences in the alkalinity of the solutions. In her later experiments, however, Norman only employed solutions in which there was "sufficient excess of chlorine to complete the reaction", that is to say a Cl/glycine ratio greater than 4·25. Her experiments were therefore limited to a study of the reactions which take place during the initial fall in available chlorine which is shown clearly in Figs. 1 and 2 of the present paper, and which was largely attributed to oxidation in the author's earlier paper. Norman's conclusions cannot be applied to the remaining portion of the curves (i.e. the secondary rise towards 100 %), where the Cl/glycine ratio falls below 1·0. Only in one instance does she give the results of an experiment in which this ratio was approached, namely in a study of the interaction of 15 mg. glycine with 14·3 mg. Cl. In an un-neutralized solution she found a reduction of about 10 % in the available chlorine within 10 hours, the equivalent figure recorded by the author for a slightly more alkaline solution being 4 %. In a neutralized solution she found a reduction of about 30 % within the same period. Commenting on these facts she states that "these results may be more reasonably explained on the basis of a slow oxidation rather than as the breakdown of an unstable dichloro-compound, which would be expected to be more stable in the neutralized experiment than in the untreated experiment". Such an assumption is quite unwarranted without experimental evidence regarding the relative stability of dichloroamino-derivatives in solutions of varying *p*H. From the results obtained in the present paper it would, in fact, appear that the dichloroamino-derivative of glycine (if formed) is more stable at *p*H 8·8 than at any *p*H on either side of this value.

In view of Norman's criticisms of the conclusions set out in the author's earlier paper, it appears desirable to summarize briefly the main facts which indicate that hypochlorites normally react with amino-acids and proteins to form (if only as unstable intermediate products) typical chloroamino-compounds.

(1) It does not appear possible to reconcile a purely oxidation theory with the fact that large quantities of amino-acids or proteins cause a smaller reduction in available chlorine than small quantities.

(2) Such anomalous behaviour can be readily explained on the assumption that hypochlorite normally acts as a chlorinating agent, and that it is only in the presence of a relative excess of hypochlorite that oxidation takes place.

(3) The fact that the secondary rise in each of the amino-acid-hypochlorite curves reaches a maximum at the theoretical point at which a definite chloroamino-derivative would be formed provides strong indirect evidence for the existence of such derivatives, though these may vary markedly in their stability [Wright, 1926].

(4) The evidence presented in the present paper confirms and amplifies these conclusions. In particular the variations observed in the quantities of glycine required to destroy the available chlorine at different *p*H show clearly that in neutral and moderately acid solutions some intermediate reaction accompanies oxidation.

(5) With cystine such differences were accompanied by visible changes in the solutions, chlorine being evolved in acid solution and polysulphides being formed in extreme alkaline solution.

(6) Under certain conditions it was found possible to isolate a yellow precipitate from the hypochlorite-protein solutions which was capable of liberating iodine from KI, presumably through the presence of chloroamino-groups in the precipitated material. In this connexion Norman gives the details of an experiment designed to determine the extent of formation of chloroamino-groups in treated gelatin. She immersed 0·2 g. of solid gelatin in a solution containing 60 mg. of available chlorine and determined the available chlorine in the gelatin after washing until the washings gave no test for chlorine. Such a technique is open to numerous errors. It is somewhat surprising to find that in the present work the ratio of gelatin/glycine used by Norman (i.e. 3·6) was precisely that at which the yellow precipitate referred to above separated out (i.e. 0·050/0·014, or 3·3). It seems probable that Norman's solutions were too alkaline, since the curves shown in Fig. 5 of this paper show that in alkaline solutions the reduction in available chlorine is at a maximum when the gelatin/chlorine ratio is about 3·3.

(7) The views put forward by the author are in conformity with those of Dakin [1915], and of Rideal [1910] who concludes that in ordinary disinfection hypochlorites leave behind them, as substitution compounds which maintain the germicidal powers, the chloroamines and chloro-proteins.

Conclusions based on indirect methods of investigation, such as reaction velocity studies, are admittedly open to criticism. The relative instability of the chloroamino-derivatives of amino-acids and proteins makes their isolation and study a matter of the utmost difficulty. Until such studies have been undertaken any final interpretation of reaction velocities will not be possible. Nevertheless the evidence summarized above does appear to provide strong grounds for postulating the formation of chloroamino-derivatives as intermediate products in the oxidation of amino-acids and proteins by hypochlorites, except in solutions of extreme alkalinity.

SUMMARY.

1. The reactions occurring between sodium hypochlorite and various amino-acids and proteins have been investigated, using buffered solutions as a means of controlling the pH.

2. The profound influence of acidity and alkalinity on the nature of the reactions has been demonstrated. In general it is found that acidity favours chlorination and alkalinity oxidation.

3. The bearing of the results on current theories of hypochlorite action is discussed.

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CCXXXVII. CHOLINE ESTER FORMATION IN, AND CHOLINE ESTERASE ACTIVITIES OF, TISSUES *IN VITRO*.

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The physiological importance of acetylcholine in the nervous system has made it very desirable to secure further knowledge of the details of acetylcholine metabolism in the body. Little is known as yet of the mode of production of this choline ester in the tissues. Beznak [1934] has reported that the ester is formed in minced frog heart muscle when sufficient eserine is present to prevent its destruction by choline esterase. Chang [1935] states that it is formed *in vitro* from placenta. Much more is known [Plattner & Galehr, 1928; Ammon & Voss, 1935; Stedman & Stedman, 1935, 1] of the breakdown of acetylcholine by an esterase which has recently been isolated and purified by Stedman & Stedman [1935, 2]. The reader is referred to summaries of recent literature on the subject in articles by Gaddum [1935] and by Ammon [1935].

Experiments have been undertaken by the authors to determine whether acetylcholine is formed as a normal product of metabolism of tissues examined *in vitro* by the Warburg manometric technique, and the following paper is concerned with a description of the results which have been found so far.

Technique. Thin slices of tissue were cut from the organs freshly dissected from the animal and placed in a manometric vessel containing Ringer solution or a phosphate saline solution. The vessel was filled with oxygen, and determinations of the oxygen consumption of the tissue were made, using the well-known manometric methods of Warburg. The experimental period varied from 1 to 3 hours at 37° or at 38·4°, at the termination of which the solution in the manometric vessel was tested for its acetylcholine activity, and the tissue slices were removed, washed, dried and weighed.

Media. Solutions were all made up so as to have an osmotic pressure approximately equal to 0·16 M NaCl. The final volume of the medium was made up to 3·0 ml. with 0·16 M NaCl after all other desired substances had been added. The hydrogen ion concentration was maintained at pH 7·4 in the experiments to be reported here.

Tissue slices. The thickness of the slices varied from one experiment to another but usually lay between 0·2 and 0·4 mm. Several slices were employed, the total dry weight in each vessel varying from 10 to 20 mg.

Neutralization of acids. All acids were neutralized before use, solutions of sodium salts being prepared.

Estimation of choline ester. The choline ester was estimated by measuring the contraction of the eserinized longitudinal muscle from the dorsum of a leech. The muscle, after dissection, was immersed at room temperature in an oxygenated saline medium of the following composition: NaCl = 0·71 %, KCl = 0·032 %, $CaCl_2$ = 0·018 %, $NaHCO_3$ = 0·012 %, glucose = 0·077 %. The final volume of medium in which the muscle was immersed was 7 ml. after the necessary additions had been made to it. 0·1–0·5 ml. of the solution from the manometric vessel was added to the medium in which the muscle was suspended, the amount added varying according to the activity of the solution in producing a contraction. Mixing was secured by the steady bubbling of oxygen through the medium. A number of contractions due to varying quantities of the experimental solution was recorded on a smoked drum by a lever to which the leech muscle was attached. These contractions were compared with those made by the addition of known quantities of acetylcholine chloride. A process of bracketing was employed to obtain the concentration of acetylcholine which on addition to the leech muscle produced a contraction most closely resembling that brought about by the experimental solution. Each contraction was allowed to take place for 3 min., after which the muscle was well washed.

Before commencing an estimation of choline ester, the leech muscle was allowed to stand, slightly stretched, in the oxygenated saline medium for 3–4 hours. Eserine (0·01 mg.) was added to it and the muscle was again allowed to stand for 15–20 min., after which it was washed with the saline medium. Its activity was then tested with known quantities of acetylcholine (e.g. 0·05–0·025 γ).[1] and its contractions were recorded, the muscle being well washed after each contraction and allowed to resume its initial length before the next contraction was obtained. Known quantities of the experimental solution were then added, the muscle being washed as before, and allowed fully to recover, after each contraction. Finally, known quantities of acetylcholine were again added and the contractions measured.

In this way it was possible to determine with fair accuracy the activity of the experimental solution in terms of acetylcholine chloride. The experimental error may be taken to be of the order of 10%.

Notation. In accordance with the usual notation the respiration Q_{O_2} is defined as the number of μl. of oxygen (reduced to N.T.P.) absorbed per mg. dry weight of tissue per hour.

The symbol R_{ChE} is introduced to denote the rate of formation of choline ester. It is defined as the amount of choline ester produced in 1 hour by 100 mg. dry weight of tissue and is expressed in equivalents of γ (10^{-3} mg.) of acetylcholine chloride.[2]

The following is an example of the manner in which the R_{ChE} of a tissue was estimated. 0·25 ml. of the experimental solution produced a contraction of the eserinized leech muscle, the dimensions of which lay midway between those of the contractions given by 0·04 γ and 0·05 γ acetylcholine chloride. This figure could be reproduced at different times in the same leech muscle. Hence 0·25 ml. of the experimental solution contained choline ester equivalent to approximately 0·045 γ acetylcholine chloride. Therefore the total quantity present in the manometric vessel was equivalent to $\frac{0\cdot045\times3}{0\cdot25}\gamma=0\cdot54\gamma$ acetylcholine chloride, since the total volume of experimental solution was 3 ml. This was formed in 3 hours by 13·6 mg. dry weight tissue. Hence $R_{\text{ChE}}=\frac{0\cdot54\times100}{3\times13\cdot6}=1\cdot3$.

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[1] A stock acetylcholine preparation (obtained as acetylcholine chloride from Messrs Hoffmann La Roche, Ltd.) was prepared in 5% NaH_2PO_4 solution. From this stock solution all the high dilutions were made. To the final dilutions of acetylcholine was added eserine sulphate (1/7000), to correspond with the concentration of eserine in the experimental solutions.

[2] To obtain R_{ChE} in terms of equivalents of the base acetylcholine, the recorded values should be multiplied by the factor 0·80.

Formation of a substance resembling acetylcholine by brain cortex slices in vitro. When rat or guinea-pig brain cortex slices are immersed in an oxygenated medium containing glucose (0·16 %), phosphate (*M*/30) and saline, and allowed to take up oxygen at 37° for 3 hours, the resultant solution, after removal of the slices, has no effect whatever on the eserinized leech muscle preparation. If, however, there is added to the medium at the commencement of the experiment eserine sulphate at a concentration of about 1 in 7000, the resultant solution is strongly active and causes a large contraction of the leech muscle preparation.

The eserine appears not to affect the respiration of the brain slices. Thus in one experiment with guinea-pig brain it was found that in the absence of eserine $R_{\mathrm{ChE}}=0$, $Q_{\mathrm{O}_2}=9{\cdot}0$ (average over 3 hours) and that in the presence of eserine $R_{\mathrm{ChE}}=1{\cdot}3$, $Q_{\mathrm{O}_2}=9{\cdot}0$ (average over 3 hours). Under no circumstances yet has it been found that a contraction of the eserinized leech muscle takes place on addition of the solution, in which metabolism of tissue slices has been allowed to occur, unless eserine was added to the solution at the beginning or near the beginning of the experiment. The conclusion would be that the presence of eserine is necessary for the appearance of the active substance in question. This fact, in itself, makes it likely, as Chang & Gaddum [1933] point out, that the active substance is a choline ester whose normal destruction by an esterase in the tissue is inhibited by the eserine. Moreover, the active substance is most unstable in alkaline solution. Thus in one experiment with rat brain cortex in a phosphate-glucose-eserine-saline medium the activity of the medium, after 3 hours at 38·4°, was such that 0·1 ml. produced a contraction equal to that given by 0·035 γ acetylcholine chloride ($R_{\mathrm{ChE}}=3{\cdot}0$). The remaining part of the medium (i.e. 2·9 ml.) was treated with 1 ml. *N* NaOH for 30 min. at room temperature. It was neutralized with 1 ml. *N* HCl and its activity tested on the leech muscle preparation. 0·5 ml. of the solution then gave no measurable contraction of the leech muscle. It was clear that the mild treatment with dilute alkali was sufficient to remove the entire activity of the medium.

The action of the substance on the eserinized leech muscle preparation has been found, like that of acetylcholine, to be unaffected by atropine. Its action, like that of acetylcholine, is diminished or abolished by the presence of morphine.

If 0·5 ml. 1/1000 morphine tartrate solution be placed in the medium in which the eserinized leech muscle is suspended, it is found that no contraction occurs on the addition of 0·05 γ acetylcholine. The addition of a further 0·05 γ acetylcholine brings about a slight contraction, which is much increased by still further additions of the acetylcholine. On washing the leech muscle preparation, the addition of 0·05 γ acetylcholine brings about its normal effect. A precisely similar phenomenon occurs when the experimental solution containing the active substance is substituted for the acetylcholine. The presence of 0·5 ml. 1/1000 morphine tartrate solution prevents the contractions due to an amount of the active substance corresponding to 0·05 γ acetylcholine, the addition of further quantities of the substance causing a contraction to appear. Reversibility is secured by washing the muscle.[1]

Although the facts stated make it probable that the active substance in the medium is an ester of choline, there is no evidence to indicate that the ester is acetylcholine. We propose therefore not to commit ourselves further than to refer to the active substance as a choline ester. The final proof that the substance in question is, in truth, a choline ester must await its isolation.

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[1] This effect of morphine will be described at length elsewhere by J. H. Q. and M. T.

Choline ester formation by brain slices. The first problem was to decide whether the choline ester found in the medium, after brain slices had been shaken in it for 3 hours at 37° was due simply to washing out of preformed ester from the tissue or whether it was formed in the course of metabolism of the tissue *in vitro*.

It has been known for some time that brain and other tissues contain an acetylcholine-like substance, and the amounts in extracts of these tissues have been estimated. Chang & Gaddum [1933] give a value of 0·4γ of acetylcholine equivalent in 1 g. of dog brain. Kwiatkowski [1935] gives the following values: 1·5–3·8γ in 1 g. dog brain, 0·4–2γ in 1 g. cat brain, 0·6–2·3γ in 1 g. guinea-pig brain. Dikshit [1934] gives 0·2–0·3γ in 1 g. basal ganglia of the cat and 0·04–1·0γ in 1 g. basal ganglia of the rabbit. Results of a similar order are given by Barsoum [1935].

The amount to be anticipated, therefore, as being due to complete removal from, say, 15 mg. dry weight of guinea-pig brain, should be (taking Kwiatkowski's figures) of the order $(0{\cdot}6\text{–}2{\cdot}3)\times\frac{15}{200}\gamma=0{\cdot}045$ to $0{\cdot}17\gamma$ (taking 200 mg. as being approximately the dry weight of 1 g. guinea-pig brain). Now the average amount of choline ester (i.e. acetylcholine equivalent) found in a medium, after metabolism in it of guinea-pig brain cortex (15 mg. dry weight) under optimal conditions, has been about 0·7γ and in the case of rat brain cortex about 1·2γ. Taking into account the fact that slices of brain cortex have been used, these figures appear to be too high to be accounted for by simple diffusion of preformed ester into the medium.

A more conclusive answer to the problem is provided by an experiment making use of the known fact that choline esterase exists in relatively large quantity in the brain [Stedman & Stedman, 1935, 1: Ammon & Voss, 1935]. This fact we have confirmed and it will be reported upon later in this paper.

The amount of choline esterase in brain cortex is far more than sufficient to decompose the preformed choline ester in the tissue in 30 min. at 37°, the assumption being made that the tissue choline ester is attacked by choline esterase at a rate comparable with the rate of hydrolysis of acetylcholine. Making a calculation from the rate of breakdown of acetylcholine chloride by cat brain cortex found by Stedman & Stedman [1935, 1], it appears that 15 mg. dry weight of the tissue will remove 525γ acetylcholine chloride in 15 min. at 30°. We have found comparable figures for rat and guinea-pig brain cortex (see Table XIII). Since the total quantity of choline ester likely to exist preformed in the brain is of the order of 0·2γ (for 15 mg. dry weight), it is obvious that there is sufficient esterase present to bring about its destruction at 37° in a few seconds.

Accordingly experiments were carried out in which eserine was added to the tissue in a phosphate-saline-glucose medium 30 min. after respiration in the Warburg manometric apparatus had commenced. The eserine was poured into the main vessel, in which the tissue was being shaken, from the side tube of the vessel. All experiments showed that the rate of choline ester formation, after this late addition of eserine, was of the same order as, but usually a little less than, when eserine was present in the medium from the start. A typical result is as follows:

Exp.		R_{ChE}
1 (*a*)	Rat brain. O_2. Phosphate-saline-glucose. Eserine	3·0
1 (*b*)	Rat brain. O_2. Phosphate-saline-glucose. Eserine added 30 min. after start of experiment	2·5

A number of estimations of R_{ChE} obtained with tissues to which eserine was added 30 min. after the commencement of each experiment will be recorded below.

Finally it should be pointed out that if experiments are carried out anaerobically (in nitrogen) the choline ester formation falls to a very low value. Thus in one experiment rat brain cortex slices in a phosphate-glucose medium gave a value of $R_{\mathrm{ChE}} = 4{\cdot}2$ in the presence of oxygen, and a value of $R_{\mathrm{ChE}} = 0{\cdot}4$ in the presence of nitrogen, this result being typical of those of a number of experiments. If the entire choline ester were preformed and simply diffused from the slices into the surrounding medium, it would be difficult to understand why the change from aerobic to anaerobic conditions should cause such a diminution in the R_{ChE}. Keeping brain slices under anaerobic conditions might, on the contrary, be expected to increase R_{ChE} by an increase in cell permeabilities due to autolytic changes.

The facts, taken as a whole, indicate that choline ester formation in tissue slices takes place as a result of metabolic changes in the tissues, and not more than a small fraction of the choline ester found can be attributed to preformed choline ester in the tissues.

We turn, now, to a more detailed consideration of the rate of choline ester formation in brain cortex slices.

Variation of R_{ChE} *with time.* When estimations are made of the rate of choline ester formation in brain slices for various periods of time at 37° or 38·4° it is found, in the case of the rat, that the greatest rate occurs in the first hour. The rate falls off appreciably after this time. Calculations of R_{ChE} are shown in Table I. The values of R_{ChE} are averages over the experimental periods stated. Such a decrease in rate may be expected if a part of the choline ester accumulating in the first hour is due to diffusion of preformed ester from the tissue slices into the medium. The same phenomenon occurs, however, if eserine is added 30 min. after the commencement of the experiment (see Exp. 5, Table I). The conclusion must be drawn therefore that the rate of choline ester formation, in rat brain cortex slices does not remain constant during the whole course of the experiment, but is most rapid at its commencement.

Table I. R_{ChE}. *Brain cortex slices.*

Variation with time. Eserine sulphate = 1/7000.

				Time from commencement of exp.		
Exp.	Animal	Temp. ° C.		1 hour	2 hours	3 hours
1	Guinea-pig	37·0	Ringer-bicarbonate* solution. 95% O_2 : 5% CO_2. Eserine	0·9	1·0	—
2	Rat	38·4	Phosphate-saline-glucose.† O_2. Eserine	5·5	2·9	3·2
3	Rat	38·4	Phosphate-saline-glucose.† O_2. Eserine	4·5	2·8	2·2
4	Rat	38·4	Phosphate-saline-glucose.† O_2. Eserine	5·3	3·5	3·5
5	Rat	38·4	Phosphate-saline-glucose.† O_2. Eserine added 30 min. after start of experiment	4·3	2·8	2·7

* Ringer-bicarbonate solution: $NaHCO_3 = 0{\cdot}025\ M$; $KCl = 0{\cdot}005\ M$; $CaCl_2 = 0{\cdot}0025\ M$; $KH_2PO_4 = 0{\cdot}001\ M$; $MgSO_4 = 0{\cdot}0012\ M$; $NaCl = 0{\cdot}11\ M$.

† This medium = sodium phosphate buffer (pH 7·4) 0·03 M; $KCl = 0{\cdot}002\ M$; $CaCl_2 = 0{\cdot}001\ M$; $NaCl = 0{\cdot}13\ M$; glucose = 0·02 M.

Dependence of choline ester formation on aerobic conditions. It has been already mentioned that the rate of choline ester formation in brain cortex slices is greatly dependent on aerobic conditions. This is shown clearly by the results given in Table II. Anaerobic conditions were secured by passing nitrogen through the manometric vessel in the inner tube of which was a small stick of damp yellow phosphorus. Potash (0·2 ml. 6 %) was placed in a side tube of the vessel. During the course of the experiment no movement of the levels of the manometric fluid occurred, indicating no absorption of gas. In a bicarbonate-glucose-Ringer medium in an atmosphere of 95% N_2 + 5% CO_2 there was also little or no formation of choline ester. When eserine was added to the medium under anaerobic conditions (by pouring in from a side tube of the manometer vessel) 30 min. after commencement of the experiment, not more than the slightest trace of choline ester could be detected. This was scarcely measurable after 2½ or 3 hours' shaking of the cortex slices at 38·4° (see Exps. 4 and 5, Table II). It seems reasonable to suppose that the small value of R_{ChE} which is found with rat brain cortex slices under anaerobic conditions when eserine is present in the medium from the start of the experiment is due to washing out of preformed choline ester from the slices. The amount found is of the same order as that to be expected from the values, already quoted, of choline ester in brain extracts.

Table II. R_{ChE} *of rat brain cortex.*

Phosphate 0·03 *M*. NaCl 0·13 *M*. Eserine = 1/7000. Glucose 0·02 *M*.

Exp.	Medium	Time hours	Temp. ° C.	O_2	N_2
1	Phosphate-saline*-eserine-glucose	3	37	1·8	0·4
2	Phosphate-saline-eserine-glucose	2·5	38·4	4·2	0·4
3	Phosphate-saline-eserine-glucose	2·5	38·4	3·0	0·9
4	Phosphate-saline-glucose. Eserine added 30 min. after commencement of exp.	2·5	38·4	2·5	0·0
5	Phosphate-saline-glucose. Eserine added 30 min. after commencement of exp.	2·5	38·4	3·0	0·0

* K^+ and Ca^{++} were absent from the medium in this experiment. In the others K^+ = 0·002*M*, Ca^{++} = 0·001 *M*.

Influence of the ionic environment on R_{ChE}. The R_{ChE} of rat brain cortex slices examined in a phosphate-sodium chloride medium is usually about the same value as that in a bicarbonate-sodium chloride medium,[1] organic metabolites being absent from these media; a few experiments have shown that a similar result applies to guinea-pig brain cortex. The effects of the addition of K and Ca ions on the two media are, however, different. Results with rat brain cortex are given in Table III. It will be seen that the low value of R_{ChE} found in a phosphate-sodium chloride medium is scarcely affected by the addition of K and Ca ions (Exp. 4). In bicarbonate media where the R_{ChE} is a little higher there is a definite increase in the rate of choline ester formation when K, or a mixture of K and Ca, ions are added (Exps. 2, 3, 4). Another difference between the rates of choline ester formation, depending on whether phosphate or bicarbonate media are used, will be noted when we turn to the consideration of the effects of glucose.

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[1] In bicarbonate (0·025 *M*) media the atmosphere in the manometer is composed of a mixture of 95% O_2 and 5% CO_2; in media containing no bicarbonate pure oxygen is present and the CO_2 formed during metabolism is absorbed by potash (on filter-paper) in the central tube of the manometer vessel.

Table III. *Rat brain cortex.*

Eserine = 1/7000. 38·4°. NaCl 0·13 M.

Exp.	Medium	R_{ChE}
1. (*a*)	Phosphate buffer 0·03 M. NaCl. $\mathrm{K^+}$ and $\mathrm{Ca^{++}}$ absent	0·6
(*b*)	Ringer-bicarbonate solution*	1·2
2. (*a*)	0·025 M $\mathrm{NaHCO_3}$. NaCl. KCl absent	0·9
(*b*)	0·025 M $\mathrm{NaHCO_3}$. NaCl. KCl 0·005 M	1·3
(*c*)	0·025 M $\mathrm{NaHCO_3}$. NaCl. KCl 0·005 M. $\mathrm{CaCl_2}$ 0·0025 M	1·1
3. (*a*)	0·025 M $\mathrm{NaHCO_3}$. NaCl	1·1
(*b*)	0·025 M $\mathrm{NaHCO_3}$. NaCl. 0·002 M KCl. 0·001 M $\mathrm{CaCl_2}$	1·7
(*c*)	0·025 M $\mathrm{NaHCO_3}$. NaCl. 0·002 M KCl. 0·001 M $\mathrm{CaCl_2}$. 0·001 M $\mathrm{MgSO_4}$	1·9
4. (*a*)	Phosphate buffer 0·03 M. NaCl	0·8 $Q_{\mathrm{O_2}}$ (3 hr.) = 4·0
(*b*)	Phosphate buffer 0·03 M. NaCl. 0·002 M KCl + 0·001 M $\mathrm{CaCl_2}$	0·7 $Q_{\mathrm{O_2}}$ (3 hr.) = 3·2
5. (*a*)	0·025 M $\mathrm{NaHCO_3}$. NaCl	1·0
(*b*)	0·025 M $\mathrm{NaHCO_3}$. NaCl. 0·002 M KCl. 0·001 M $\mathrm{CaCl_2}$	1·4
(*c*)	0·025 M $\mathrm{NaHCO_3}$. NaCl. 0·004 M KCl. 0·002 M $\mathrm{CaCl_2}$	1·4

* Composition given under Table I.

The effects of glucose on choline ester formation in brain slices. In phosphate media the presence of glucose accelerates greatly the rate of choline ester formation in brain cortex slices. This is shown by a few typical results shown in Table IV. The effect of the glucose with rat brain is to increase R_{ChE} four- or five-fold, and its effect is still large in the absence of added potassium and calcium ions (see Exp. 1). The effect of glucose is equally great when eserine is added to the medium 30 min. after the commencement of the experiments. When we turn however to bicarbonate media we note the following phenomena.

Table IV. *Rat brain cortex.*

Eserine = 1/7000. Phosphate = 0·03 M. NaCl = 0·13 M.

Exp.	Medium	Time hours	Temp. °C.	R_{ChE}
1. (*a*)	Phosphate-NaCl	3	37	0·7
(*b*)	Phosphate-NaCl-glucose (0·01 M)	3	37	2·8
2. (*a*)	Phosphate-NaCl-KCl (0·002 M)-$\mathrm{CaCl_2}$ (0·001 M)	3	38·4	0·7
(*b*)	Phosphate-NaCl-KCl (0·002 M)-$\mathrm{CaCl_2}$ (0·001 M)-glucose (0·01 M)	3	38·4	2·9
3. (*a*)	Phosphate-NaCl-KCl (0·002 M)-$\mathrm{CaCl_2}$ (0·001 M)	3	38·4	1·1 $Q_{\mathrm{O_2}}$ (av. 3 hr.) = 4·1
(*b*)	Phosphate-NaCl-KCl (0·002 M)-$\mathrm{CaCl_2}$ (0·001 M)-glucose (0·02 M)	3	38·4	3·2 $Q_{\mathrm{O_2}}$ (av. 3 hr.) = 11·6
4. (*a*)	Phosphate-NaCl-KCl (0·002 M)-$\mathrm{CaCl_2}$ (0·001 M)	3	38·4	0·7 $Q_{\mathrm{O_2}}$ (av. 3 hr.) = 3·2
(*b*)	Phosphate-NaCl-KCl (0·002 M)-$\mathrm{CaCl_2}$ (0·001 M)-glucose (0·02 M)	3	38·4	3·6 $Q_{\mathrm{O_2}}$ (av. 3 hr.) = 10·8

The addition of glucose to a bicarbonate medium containing no added K or Ca ions results in a large acceleration in the rate of choline ester formation. The R_{ChE} may rise from 0·4 to 2·2 (see Exp. 1, Table V). The further addition of K and Ca ions to a bicarbonate-glucose medium lowers the R_{ChE} (Exps. 2 and 3, Table V). The addition of glucose to a bicarbonate-Ringer medium has but a small effect on the rate of choline ester formation (Exps. 1 and 4, Table V). With guinea-pig brain cortex we have noticed that the presence of glucose usually diminishes the R_{ChE} found in a bicarbonate-Ringer medium.

Table V. *Rat brain cortex.*

Time, 3 hours. Temp. 38·4°. Eserine 1/7000. 95% O_2 + 5% CO_2.

Exp.		Medium	R_{ChE}
1.	(*a*)	0·025 *M* $NaHCO_3$. NaCl	0·4
	(*b*)	0·025 *M* $NaHCO_3$. NaCl-glucose (0·01 *M*)	2·2
	(*c*)	Ringer*-bicarbonate	1·3
	(*d*)	Ringer*-bicarbonate-glucose (0·01 *M*)	1·6
2.	(*a*)	0·025 *M* $NaHCO_3$. 0·13 *M* NaCl. 0·02 *M* glucose	3·7
	(*b*)	0·025 *M* $NaHCO_3$. 0·13 *M* NaCl. 0·002 *M* KCl. 0·001 *M* $CaCl_2$. 0·02 *M* glucose	2·2
3.	(*a*)	0·025 *M* $NaHCO_3$. 0·13 *M* NaCl. 0·02 *M* glucose	2·3
	(*b*)	0·025 *M* $NaHCO_3$. 0·13 *M* NaCl. 0·002 *M* KCl. 0·001 *M* $CaCl_2$. 0·02 *M* glucose	1·4
	(*c*)	0·025 *M* $NaHCO_3$. 0·13 *M* NaCl. 0·004 *M* KCl. 0·002 *M* $CaCl_2$	1·4
4.	(*a*)	Ringer*-bicarbonate	1·5
	(*b*)	Ringer*-bicarbonate-glucose (0·16 %)	1·3

* Composition given under Table I.

The reasons for these phenomena are as yet obscure, but attention should be drawn to the fact that the aerobic glycolysis by brain slices in a bicarbonate-glucose medium is always much greater in the absence of potassium and calcium ions than in their presence [Dickens & Greville, 1935].

Turning once more to the consideration of phosphate-glucose media we find that the addition of potassium and calcium ions has the reverse effect to that happening in a bicarbonate-glucose medium, i.e. the R_{ChE} is increased. Typical results are shown in Table VI.

Table VI. *Rat brain cortex.*

Time 3 hours. Temp. 38·4 . Eserine 1/7000. O_2. Phosphate = 0·03 *M*. NaCl = 0·13 *M*.

				Q_{O_2}	
Exp.		Medium	R_{ChE}	First 30 min.	3 hr. (av.)
1.	(*a*)	Phosphate-NaCl-glucose (0·02 *M*)	1·8	15·3	7·5
	(*b*)	Phosphate-NaCl-glucose + KCl (0·002 *M*), $CaCl_2$ (0·001 *M*)	3·6	12·8	10·8
2.	(*a*)	Phosphate-NaCl-glucose (0·02 *M*)	1·9	16·0	8·5
	(*b*)	Phosphate-NaCl-glucose + KCl (0·002 *M*), $CaCl_2$ (0·001 *M*)	2·2	15·3	11·6
	(*c*)	Phosphate-NaCl-glucose + KCl (0·008 *M*), $CaCl_2$ (0·004 *M*)	2·6	14·6	12·6
3.	(*a*)	Phosphate-NaCl-glucose (0·01 *M*)	2·6	15·8	9·7
	(*b*)	Phosphate-NaCl-glucose (0·01 *M*) + KCl (0·002 *M*)	4·3	—	—
	(*c*)	Phosphate-NaCl-glucose (0·01 *M*) + KCl (0·004 *M*)	4·0	14·0	12·1
4.	(*a*)	Phosphate-NaCl-glucose (0·02 *M*)	3·0	—	—
	(*b*)	Phosphate-NaCl-glucose (0·02 *M*) + KCl (0·002 *M*)	4·1	—	—
5.*	(*a*)	Phosphate-NaCl-glucose (0·02 *M*)	2·0	—	—
	(*b*)	Phosphate-NaCl-glucose (0·02 *M*) + KCl (0·002 *M*) + $CaCl_2$ (0·001 *M*)	2·7	—	—

* In this case eserine was added 30 min. after commencement of the experiment.

The effects of addition of K and Ca ions are not very regular, but they are always in the same direction—that of an increase in the R_{ChE}. It should be noted that in a phosphate-glucose medium to which no K and Ca ions have been added the Q_{O_2} falls rapidly during the experimental period. In the presence of K and Ca ions the Q_{O_2} remains comparatively steady over a long period (see Exps. 1, 2 and 3, Table VI). This phenomenon has been recently observed by Dickens & Greville [1935].

There is little doubt that the presence of K and Ca ions markedly influence the course of glucose metabolism in tissue slices. They also affect choline ester formation in brain cortex slices in glucose media in such a way that R_{ChE} is high when aerobic glycolysis is high (bicarbonate, absence of K and Ca) and when respiration is high (phosphate, presence of K and Ca). It is reasonable to suggest that there is a definite link between the two metabolic processes, one involving glucose breakdown and the other choline ester formation.

Values of R_{ChE} calculated as averages from the results of a number of experiments are given in Table VII. These were obtained with rat brain cortex slices in various media in the presence and absence of glucose.

Table VII. R_{ChE} *(average values).*

Eserine = 1/7000. 38·4°.

Medium	Glucose absent	Glucose present
0·025 *M* $NaHCO_3$ + 0·13 *M* NaCl	0·7	2·7
0·025 *M* $NaHCO_3$ + 0·13 *M* NaCl + 0·002 *M* KCl + 0·001 *M* $CaCl_2$	1·4	1·6
0·03 *M* phosphate + 0·13 *M* NaCl	0·8	2·0
0·03 *M* phosphate + 0·13 *M* NaCl + 0·002 *M* KCl + 0·001 *M* $CaCl_2$	0·9	2·9

Effects of metabolites other than glucose on R_{ChE}. The addition of sodium lactate (*dl*) or of sodium pyruvate to a phosphate-saline medium increases R_{ChE} with rat brain cortex slices, but the rate of increase of choline ester formation is not usually as great as with glucose. Representative results are given in Table VIII. The increase occurs, as with glucose, when eserine is present at the start of the experiment or when it is added 30 min. after the commencement of the experiment. The increase occurs whether K and Ca ions have been added or not.

Table VIII. *Rat brain cortex.*

Variations of R_{ChE} with substrate. Eserine = 1/7000. Phosphate = 0·03 *M*. NaCl 0·13 *M*.

Exp.		Medium	Time hr.	Temp. °C.	R_{ChE}	Q_{O_2} (av. 3 hr.)
1.	(*a*)	Phosphate-NaCl	3	37	0·7	3·5
	(*b*)	Phosphate-NaCl-glucose (0·01 *M*)	3	37	2·6	8·6
	(*c*)	Phosphate-NaCl-lactate (0·026 *M*)	3	37	1·8	9·4
	(*d*)	Phosphate-NaCl-succinate (0·016 *M*)	3	37	0·7	6·5
2.	(*a*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$	3	38·4	1·1	4·1
	(*b*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-glucose (0·02 *M*)	3	38·4	3·2	11·6
	(*c*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-lactate (0·02 *M*)	3	38·4	3·2	9·9
	(*d*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-pyruvate (0·02 *M*)	3	38·4	2·0	9·7
3.	(*a*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-glucose (0·02 *M*)	3	38·4	3·0	10·6
	(*b*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-succinate (0·02 *M*)	3	38·4	0·6	6·8
4.	(*a*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-glucose (0·02 *M*)	3	38·4	3·4	12·0
	(*b*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-lactate (0·02 *M*)	3	38·4	2·4	9·9
5.*	(*a*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$	2½	38·4	0·6	—
	(*b*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-glucose (0·02 *M*)	2½	38·4	2·4	—
	(*c*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-lactate (0·02 *M*)	2½	38·4	1·7	—
	(*d*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-pyruvate (0·02 *M*)	2½	38·4	1·9	—
6.*	(*a*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$	2½	38·4	0·7	—
	(*b*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-glucose (0·02 *M*)	2½	38·4	2·5	—
	(*c*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-fructose (0·02 *M*)	2½	38·4	2·3	—
	(*d*)	Phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$-galactose (0·02 *M*)	2½	38·4	1·8	—

* Eserine added 30 min. after the commencement of the experiment.

The addition of sodium succinate, however, does not result in any increased R_{ChE} (see Exps. 1 and 3, Table VIII). This fact is of considerable interest for it shows that choline ester formation does not necessarily depend on an increased respiration of the brain cortex slices. Succinate increases markedly the respiration of brain cortex, though the increase is not so great as with glucose or lactate for equivalent concentrations; these results are noted in Table VIII.

Turning to sugars other than glucose we find that fructose is as effective as glucose but that galactose is less efficient. In Exps. 5 and 6 recorded in Table VIII eserine was added 30 min. after the start of the experiments.

Two experiments have shown that the presence of sodium glutamate (0·04 *M*) results in an increase of R_{ChE}: with rat brain cortex in phosphate-saline media R_{ChE} was 1·7 (3 hours 38·4°). The increase is not as great as with glucose.

Effects of substituting α-glycerophosphate for phosphate on R_{ChE}. The substitution of 0·03 *M* sodium α-glycerophosphate (*p*H 7·4) for phosphate buffer results in an increased value of R_{ChE} for rat brain cortex. Exps. 1 and 2, Table IX, show that the value of R_{ChE} is doubled. As is well known the presence of α-glycerophosphate increases the respiration of brain. The addition of glucose to the α-glycerophosphate medium increases the R_{ChE}, the increase being of the same order as that which occurs when glucose is added to a phosphate medium.

Table IX. *Effect of α-glycerophosphate on* R_{ChE}. *Rat brain cortex.*

Time, 3 hours. Temp. 38·4°. O_2. Eserine 1/7000.

Exp.		Medium	R_{ChE}	Q_{O_2} (av. 3 hr.)
1.	(*a*)	0·03 *M* phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$	0·7	3·0
	(*b*)	0·03 *M* phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$ + glucose (0·02 *M*)	2·9	11·8
	(*c*)	0·03 *M* α-glycerophosphate-0·002 *M* KCl-0·001 *M* $CaCl_2$	1·4	8·4
	(*d*)	0·03 *M* α-glycerophosphate-0·002 *M* KCl-0·001 *M* $CaCl_2$ + glucose (0·02 *M*)	4·0	12·4
2.	(*a*)	0·03 *M* phosphate-NaCl-0·002 *M* KCl-0·001 *M* $CaCl_2$	1·1	3·8
	(*b*)	0·03 *M* α-glycerophosphate-0·002 *M* KCl-0·001 *M* $CaCl_2$	2·2	8·3
3.	(*a*)	0·03 *M* α-glycerophosphate-NaCl-0·001 *M* $CaCl_2$	1·2	7·1
	(*b*)	0·03 *M* α-glycerophosphate-NaCl-0·001 *M* $CaCl_2$ + glucose (0·02 *M*)	2·9	11·6
4.	(*a*)	0·03 *M* α-glycerophosphate-NaCl-glucose (0·02 *M*)	3·0	10·8
	(*b*)	0·03 *M* α-glycerophosphate-NaCl-glucose (0·02 *M*) + KCl 0·002 *M* + $CaCl_2$ 0·001 *M*	3·0	14·2

A few experiments have indicated that the R_{ChE} in a α-glycerophosphate-NaCl-glucose medium is not markedly affected by the inclusion of K and Ca ions. Exp. 4, Table IX, shows a typical result. As in a phosphate medium the presence of K and Ca ions decreases the rate of fall of Q_{O_2}.

Effects of addition of choline and of sodium acetate on R_{ChE}. The addition of choline (as the hydrochloride) to bicarbonate or phosphate media, in concentrations sufficiently low not to affect the leech muscle preparation when suitable volumes are taken for testing purposes, has no definite effect on the R_{ChE}. This applies to both guinea-pig and rat brain cortex slices. Typical results are shown in Table X. The amount of choline chloride added to each vessel was 100γ or 200γ. Now had only 0·5% choline ester synthesis occurred from the choline added with the production of, say, 0·5 or 1·0γ choline ester, this amount would have been easily measurable. The results show that with a concentration of choline chloride of 33γ/ml. or 66γ/ml. no perceptible synthesis of choline ester occurs. This is the case whether glucose is present or not.

The addition of sodium acetate (0·02 *M*) seems to have a small accelerating effect on R_{ChE} (Exps. 6 and 7, Table X) whether choline is present or not. It

Table X. *Brain cortex slices.*

Eserine 1/7000. Phosphate 0·03 M. NaCl 0·13 M.

Exp.	Animal	Medium	Time hr.	Temp. ° C.	R_{ChE}
1. (*a*)	Guinea-pig	Bicarbonate-Ringer*	3	37	1·7
(*b*)	,,	Bicarbonate-Ringer* + choline (100 γ)	3	37	1·8
2. (*a*)	,,	Bicarbonate-Ringer*	3	37	0·9
(*b*)	,,	Bicarbonate-Ringer* + choline (100 γ)	3	37	1·0
3. (*a*)	,,	Phosphate-NaCl-glucose	3	37	1·3
(*b*)	,,	Phosphate-NaCl-glucose-choline (100 γ)	3	37	0·0†
(*c*)	,,	Phosphate-NaCl-glucose-choline (100 γ)	3	37	1·4
4. (*a*)	Rat	Phosphate-NaCl-glucose	3	37	2·8
(*b*)	,,	Phosphate-NaCl-glucose-choline (100 γ)	3	37	3·2
5.	,,	Bicarbonate-Ringer-glucose-choline (100 γ)	3	37	1·0
6. (*a*)	,,	Bicarbonate-Ringer-glucose	3	38·4	1·0
(*b*)	,,	Bicarbonate-Ringer-glucose-choline (200 γ)	3	38·4	1·1
(*c*)	,,	Bicarbonate-Ringer-glucose-acetate (0·02 M)	3	38·4	1·6
(*d*)	,,	Bicarbonate-Ringer-glucose-acetate (0·02 M) + choline (200 γ)	3	38·4	1·7
7. (*a*)	,,	Bicarbonate-Ringer-glucose	2	38·4	1·3
(*b*)	,,	Bicarbonate-Ringer-glucose + acetate (0·02 M)	2	38·4	1·7

* Composition of Ringer is given under Table I.

† Too small to be measured; eserine omitted in this case.

seems scarcely likely that so small an effect can be taken as an indication of the synthesis in the tissue of acetylcholine from acetate ions and choline.

It would be expected that eserine which inhibits the activity of choline esterase will not only prevent hydrolysis of choline ester, but will also inhibit its synthesis by this enzyme. The fact that the additions of choline and of acetate do not bring about any marked production of acetylcholine in the tissue in the presence of eserine suggests that the choline ester which is formed in brain tissue slices in the presence of eserine does not arise from the products of its hydrolysis.

Action of poisons on R_{ChE}.

(*a*) *Cyanide.* The addition of potassium cyanide (0·06 %) to an oxygenated bicarbonate-Ringer-glucose-eserine medium reduces the R_{ChE} of rat brain cortex to the anaerobic level. A value of $R_{\text{ChE}} = 1·5$ was reduced, by the presence of cyanide, under aerobic conditions to $R_{\text{ChE}} = 0·5$. Similarly, in a phosphate-saline-glucose-eserine medium, a value of $R_{\text{ChE}} = 1·9$ was reduced to $R_{\text{ChE}} = 0·4$ by the presence of cyanide (0·06 %).

(*b*) *Sodium fluoride.* The addition of 0·01 M NaF does not appreciably affect the R_{ChE} found with rat brain cortex in a phosphate-saline-glucose-eserine medium. In one experiment the value in absence of NaF was $R_{\text{ChE}} = 3·4$ and in the presence of 0·01 M NaF R_{ChE} was found equal to 4·3. Yet in this experiment the Q_{O_2} was reduced by the fluoride from a value of 12·0 to 4·6.

Evidently even in the presence of fluoride the presence of glucose will stimulate the formation of choline ester under the conditions of our experiments.

Choline ester formation in various animal tissues. Choline ester formation has been found to be greatest with brain tissue slices. Among the tissues examined—brain, kidney, liver, spleen, testis, diaphragm and defibrinated blood—not more than traces of choline ester have been found in tissues other than brain and diaphragm. Diaphragm of the rat only has been examined and this proves

Table XI. R_{ChE} *of various organs.*

Animal	Guinea-pig	Rat
Medium	Bicarbonate-Ringer-eserine	Phosphate-saline-glucose-eserine (K, Ca present)
Organ		
Brain cortex	1·7	2·5
Kidney	<0·3	<0·3
Liver	<0·3	<0·3
Spleen	<0·3	<0·3
Testis	<0·3	<0·3
Diaphragm	—	0·8
Blood (defibrinated)	<0·3	—

to be one-third as active as rat brain cortex when examined in a phosphate-glucose medium. The results are noted in Table XI. There is little doubt that choline ester formation, so far as our experiments *in vitro* have gone, is restricted chiefly to the brain.

Acetylcholine hydrolysis by tissue slices. The hydrolysis was measured in the Warburg apparatus by pouring 0·2 ml. acetylcholine chloride solution from the side tube of the manometric vessel into the main vessel which contained tissue slices suspended in 2·8 ml. of a bicarbonate-saline solution and noting the rate of evolution of CO_2. The acetylcholine was added after temperature equilibrium (37° or 38·4°) had been attained. Air in the apparatus had been displaced by a mixture of 95% N_2 and 5% CO_2, and strict anaerobiosis was secured by placing small pieces of damp yellow phosphorus in the central tube of the manometer vessel. The solution in the main vessel consisted of 0·025 *M* $NaHCO_3$ and 0·13 *M* NaCl, together with 0·002 *M* KCl and 0·001 *M* $CaCl_2$.

As the acetylcholine was hydrolysed CO_2 was evolved from the medium, the rate of evolution being a measure of the rate of hydrolysis. A similar manometric method for following acetylcholine hydrolysis has already been used by Stedman & Stedman [1935, 1] and Ammon & Voss [1935]. The rate of hydrolysis is given in the following units:

$Q^{N_2}_{CO_2}$ (*acetylcholine*) = μl. CO_2 (reduced to N.T.P.) evolved per mg. dry weight of tissue per hour.

0·2 ml. of a solution containing 100 mg. acetylcholine chloride in 5 ml. water was placed in the side tube of the vessel. After pouring the acetylcholine into the main vessel its initial concentration became 0·0073 *M*.

With this initial concentration the rate of evolution of CO_2 and therefore the rate of hydrolysis are approximately constant until the concentration of acetylcholine reaches about 0·003 *M*, when the rate of hydrolysis begins appreciably to diminish. Typical results are shown in Table XII.

Values of $Q^{N_2}_{CO_2}$ (*acetylcholine*) are shown in Table XIII where it will be seen (*a*) with rat tissues that spleen is the most active organ in decomposing the choline ester, (*b*) with both guinea-pig and rat, kidney has feeble choline esterase activity, whilst liver and testis have activities less than that of brain cortex. The values recorded in Table XIII have not been corrected for the small blanks obtained in the absence of acetylcholine or of tissue.

The results (Tables XI, XIII) show that there is no relationship between the power of an organ to break up acetylcholine and its power to form choline ester *in vitro*.

Table XII.

Time from addition of acetylcholine t (min.)	Vol. of CO_2 evolved v (μl.)	v/t
	Rat brain.	
45	116·0	2·5
75	192·0	2·5
105	262·8	2·5
135	318·5	2·4
180	384·7	2·1
240	445·4	1·8
	Guinea-pig blood.	
45	87·0	1·9
75	150·0	2·0
105	211·7	2·0
135	271·3	2·0
165	318·5	1·9
195	354·0	1·8
240	381·5	1·5

Table XIII. *Acetylcholine hydrolysis by various organs.*

Bicarbonate-saline. 95% N_2 + 5% CO_2. Phosphorus.
Initial concentration of acetylcholine = 0·0073 *M*.
Taken over period 0–60 min.

	$Q^{N_2}_{CO_2}$ (*acetylcholine*).	
	Rat (38·4°)	Guinea-pig (37)
Brain cortex	17·8	17·3
Liver	10·1	3·1
Kidney	7·9	3·2
Spleen	26·2	6·0
Testis	12·4	2·1
Defibrinated blood	5·2	13·3

SUMMARY.

1. When brain cortex slices are allowed to respire in a medium containing eserine a substance is formed which produces a powerful contraction of an eserinized leech muscle preparation. The substance is only formed in the presence of eserine, is unstable in dilute alkaline solution and its effect on the leech muscle is counteracted, like that of acetylcholine, by the presence of morphine. It is considered to be a choline ester. It is shown that the choline ester is formed as a result of metabolic processes in the tissue slices, only a small fraction of it being due to preformed ester within the tissue.

2. The rate of choline ester formation is expressed by the symbol R_{ChE}. This represents the amount of choline ester formed in 1 hour by 100 mg. dry weight tissue and is expressed in equivalents of acetylcholine chloride.

3. R_{ChE} varies during the period of an experiment, it being greatest in the first hour.

4. Under anaerobic conditions, or under aerobic conditions in the presence of cyanide, R_{ChE} falls to a very low level. Oxygen is necessary for maintenance of R_{ChE} at a high level.

5. The presence of glucose greatly increases R_{ChE} in phosphate or bicarbonate media when potassium and calcium ions are absent. The addition of K and Ca ions decreases R_{ChE} in a bicarbonate-glucose medium and increases R_{ChE} in a

phosphate-glucose medium. Attention is drawn to the effects of these ions on glucose metabolism in bicarbonate and phosphate media. It is suggested that there is a link between glucose metabolism and choline ester metabolism in tissue slices.

6. The presence of sodium lactate, pyruvate, α-glycerophosphate or glutamate increases R_{ChE} but their effects are not as great as those of glucose. The presence of sodium succinate does not increase R_{ChE}.

7. The addition of sodium fluoride does not diminish R_{ChE} of brain in a glucose medium.

8. The addition of choline does not measurably increase R_{ChE}. Nor does the presence of a large excess of acetate ions markedly increase R_{ChE}.

9. Kidney, liver, spleen or testis has no measurable activity in forming choline ester under conditions most favourable for its production from brain. Rat diaphragm has about one-third the activity of rat brain.

10. Choline esterase activities of brain, liver, kidney, spleen, testis and defibrinated blood are recorded. There is no correlation between the choline esterase of an organ and its power of producing choline ester *in vitro*.

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CCXXXVIII. THE DIGESTIBILITY OF DIETARY PROTEIN IN THE RUMINANT.

I. ENDOGENOUS NITROGEN EXCRETION ON A LOW NITROGEN DIET AND IN STARVATION.

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In studying the digestibility of food protein the normal procedure is to analyse the food for its content of N and to feed a constant amount to experimental animals. After a preliminary period of such length that the faeces can be presumed to represent the experimental ration, the faeces are collected and analysed for a period of 7–10 days. In certain experiments markers have been used to distinguish the faeces of this period from that of the preceding and following periods. The percentage of the ingested nitrogen *not* appearing in the faeces is called the "coefficient of digestibility of protein".

This definition assumes that all the faecal N is derived from the ingested N. Previous work [Schneider, 1934] has shown, however, that the faecal N can be divided into three fractions, (*a*) a constant fraction found when the animal is fasting, (*b*) a fraction dependent on the amount of both total dry matter and of indigestible dry matter in the diet and (*c*) a fraction dependent on the nitrogen content of the diet.

METABOLIC FAECAL NITROGEN.

Considerable work has been carried out on the metabolic faecal N of the one-stomached animal and its relation to the food intake. Mitchell [1924] concluded that the metabolic faecal N of young growing rats was directly proportional to the food intake. Whilst he recognized the presence of a constant amount of endogenous N he considered it quantitatively of no importance and recommended therefore that the metabolic faecal N on a normal diet should be calculated as faecal N/100 g. food intake.

Boas-Fixsen and Jackson [1932], working with adult male rats and consequently with a much lower food intake, found that the endogenous faecal N was roughly constant for any particular rat and was by no means directly proportional to the food intake. They considered that the food intake should not be taken into account in estimating the metabolic faecal N, in the calculation of the biological value of proteins for maintenance.

Schneider [1934] made a statistical analysis of the results obtained in Mitchell's laboratory over a number of years. He confirmed Boas-Fixsen and Jackson's finding that, in an animal eating its maintenance requirements or less, the constant fraction of the metabolic faecal N represented a comparatively large fraction of the total faecal N. Calculation of the metabolic faecal N on the assumption that it is directly proportional to the food intake would thus involve an error as great as if the food intake were not taken into account at all. Where, however, the food intake is above the maintenance requirements of the animal the metabolic faecal N may be taken as directly proportional to the food intake.

Voit [1932], working with adult dogs, found that the constant fraction was by no means negligible. He concluded from his experiment that fat exercised less effect on the metabolic faecal N than carbohydrate and protein. Mitchell [1934], however, could not confirm Voit's results, but found that, irrespective of the type of food ingested, the metabolic faecal N bore a constant relationship to the food intake.

With ruminants, various investigators have attempted to obtain a figure for the metabolic N. Morgen *et al.* [1914] fed to sheep a ration consisting of extracted straw, starch, sugar and oil. In most of the experiments blood albumin was added to increase the palatability of the ration. It was thought that, since the albumin would be completely digested, it would have no effect on the faecal N excretion. This fact had previously been noted with one-stomached animals by Mendel & Fine [1912] and Underhill & Goldschmidt [1913] and was later confirmed by Mitchell [1924]. Morgen *et al.* observed 0·51 g. of faecal N excreted per 100 g. of dry matter intake. Later, Titus [1927] investigated the endogenous N excretion of steers, the rations consisting of varying amounts of alfalfa and cellomass (dry paper pulp). He noted that the faecal N varied markedly with the moisture content of the faeces. By calculating all his results on an assumed 80 % moisture content, he found, by extrapolation, that the metabolic faecal N was 0·34 g. per 100 g. dry matter intake.

In the present series of experiments an attempt has been made to obtain a value for the metabolic faecal N of goats and sheep by feeding N-free diets and rations of low N content.

I. *The effect of a nitrogen-free diet on the faecal nitrogen excretion.*

Four goats and four sheep were used, the experiments being divided into three periods, (1) starvation, (2) low food intake, (3) high food intake. The energy was provided as starch and olive oil. The olive oil comprised 2 % of the high food intake and 2–9 % of the low food intake diet. To provide roughage, 12–40 % sawdust or paper was incorporated in the diet in the experiments with low food intake. In the experiments with high food intake the diet contained 20 % of sawdust. 2–5 % of a salt mixture was given in all diets and 30 drops of adexolin daily to provide vitamins A and D. In the high food intake experiments the diet was calculated to give the maintenance energy requirements from Benedict & Ritzman's [1926] figures for sheep. The N content of the diets was 0·02 % except in three experiments with high food intake, in which there was 0·48 % caseinogen and 0·32 % urea N. These diets contained rather less indigestible carbohydrate than the average normal diet, since it was not possible to give any more cellulose. The total food intake on the high food intake diet was therefore less than normal.

It was necessary to feed the diets by a bottle and stomach tube, since the animals would not consume a N-free diet of their own accord. About half the paper of the low food intake diet was, however, voluntarily consumed by the goats. It was not possible to perform satisfactory experiments on the sheep with the high food intake diet, since the animals became ill and eventually died. Apparently the fibre accumulated in the rumen instead of being passed on to the true stomach. The experiments lasted 6–14 days. All the starvation experiments lasted 7 days, during which water was given *ad lib.* as in the other experiments. The urine and faeces were collected daily. A preliminary period was given, usually 3 or 4 days, till the animals reached a constant N excretion.

There was some doubt as to the validity of results obtained by such a drastic method of feeding. They might be too low, if the fibre were to accumulate in the rumen instead of being regurgitated and then passed into the true stomach. This occurred when the sheep were given the high food intake, so that the faecal N was actually lower than with the low food intake, while the weight of faeces was also smaller. Nevertheless, it was possible to keep the goats up to 14 days on the high food intake and 21 days on the low food intake without any ill effects.

However, in order to obtain more definite evidence on this point, an attempt was made to get some of the animals to consume a N-free diet voluntarily. After considerable difficulty it proved possible to feed to three goats a diet composed of starch, sugar, sawdust and a small quantity of straw. The amount of straw was too small to have affected the results even if it contained indigestible N. The details of these diets are shown in Table I. 10 g. of salt and 5 ml. of cod-liver oil were given to each animal daily.

Table I. *Daily rations.*

Animal	Body weight kg.	Starch g.	Sawdust g.	Oat straw g.	Sugar g.
1	18·5	114	10	101	10
2	14·2	111	17	87	10
3	24·0	154	14	95	10

The results of the two series of experiments are shown in Fig. 1, where $\frac{\text{Faecal N}}{(\text{Body weight})^{\frac{2}{3}}}$ is plotted against $\frac{\text{Food intake}}{(\text{Body weight})^{\frac{2}{3}}}$ in order to compensate for the difference in size between the different animals. The general form of the graph is in agreement with that obtained with the one-stomached animal. The constant fraction is a fairly large proportion of the total metabolic faecal N. The points corresponding to the animals which ate voluntarily (denoted by crosses) lie in the centre of the distribution of points. It appears therefore that forcible feeding did not affect the general trend of faecal N excretion.

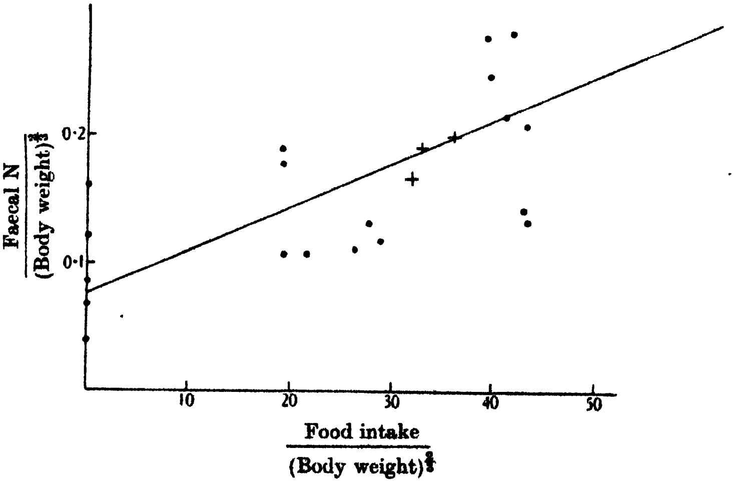

Fig. 1. Faecal N excretion of goats and sheep.

The line of the graph corresponds to a metabolic faecal N of 0·50 g. per 100 g. dry matter food intake at the high food intake level. As explained above, this point on the graph corresponds to a food intake which is below the normal. At the normal food intake the value would be 0·45, which is lower than Morgen's figure of 0·51 for the sheep. The metabolic faecal N of the goat and sheep appears then to be relatively high, being in fact about double the endogenous urinary excretion.

II. *The effect of fibre on the metabolic nitrogen excretion.*

The effect of indigestible non-nitrogenous substances on endogenous faecal N excretion has been studied with rats [Mendel & Fine, 1912; Mitchell, 1924]. It was found that there was an increase in faecal N which depended on the amount of roughage ingested. With the ruminant, fibre is a very essential part of the ration. It was important, therefore, to discover whether the effect of fibre on the metabolic faecal N excretion of the ruminant was similar to that found with rats.

Two series of experiments were carried out on goats. Three animals which had been forcibly fed on the low food intake diet in the first series of experiments were given a further experimental period in which paper was substituted for half the starch. The total food intakes were slightly different in both experiments, but this was insufficient materially to affect the results. The experimental periods were of 6–8 days' duration.

It will be seen that the fibre invariably increased the metabolic faecal N (Table II).

Table II. *The effect of fibre on the faecal nitrogen excretion.*

		Diet without fibre		Diet with fibre			
Goat	Body weight kg.	Starch etc. (dry) g.	Paper (dry) g.	Starch etc. (dry) g.	Paper (dry) g.	Faecal N without fibre (g.) 100 g. dry food intake	Faecal N with fibre (g.) 100 g. dry food intake
4	63	366	0	323	63	0·505	0·585
5	23	147	0	135	23	0·490	0·550
6	39	262	0	226	72	0·410	0·450

A more accurate series of experiments was attempted on three further goats. The periods of collection in these experiments were 10 days, exclusive of the preliminary period. Paper and cellophane were used as the source of fibre and it was found possible to make a greater change in the fibre content of the two diets.

It will be seen (Table III) that animal 6 had a completely N-free diet while the small quantity of maize and oats in the diets of the other two animals was not sufficient to affect the results even if the digestibility had fallen slightly in the high-fibre period.

Table III.

		Low-fibre diet			High-fibre diet		
Goat	Body weight kg.	Oats and flaked maize g.	Paper g.	Starch etc. g.	Oats and maize g.	Paper and cellophane g.	Starch etc. g.
5	23	30	10	173	30	101	82
3	24	30	10	114	30	82	48
6	38	0	23	236	0	126	144

As in the first series of experiments, the additional fibre increased the metabolic faecal N of each animal (Table IV). It is possible that the large increase noted would have been reduced on a higher food intake. This, however,

Table IV.

Goat	Faecal N on low-fibre diet g.	Faecal N on high-fibre diet g.	Difference in fibre content of 2 diets g.	Increase in faecal N/100 g. fibre g.
5	1·10	1·84	75	1·77
3	0·87	1·09	67	0·59
6	1·24	1·78	100	0·97
			Mean	1·11

proved impossible to investigate, since the animals did not maintain their appetites when more food was given.

Since the effect of fibre on the metabolic faecal N was so great it seemed possible that the true digestibility of straw, which contains 35% of fibre, might be higher than has been generally supposed. If this were so, straw might be used as roughage in determining the metabolic faecal N of the cow, which will not consume cellophane or paper. Two of the goats which ate voluntarily, in a previous experiment (p. 1684), the diet containing starch, sugar, sawdust and a little straw, were therefore given a diet composed only of straw with the same quantity of salts and cod-liver oil.

It appears from the results (Table V) that the true digestibility of straw, using the goat as experimental animal, is 100%. It must, however, be remembered that the experimental error involved in the measurement of small differences in faecal N is large. Also the number of experiments is small. Nevertheless it can be concluded with safety that the true digestibility of straw is high. This would explain the remarkably close agreement between the results of the present series of experiments and those of Morgen, who fed extracted straw in the diet.

Table V. *The digestibility of straw.*

Goat		Daily intake g.	N ingested g.	Faecal N g.	Difference in straw intake g.	Difference in fibre intake g.	Difference in faecal N g.	Difference in met. faecal N (due to difference in fibre intake) g.
1	Low-straw	114 starch + sugar 10 sawdust 101 straw	0·31	1·16	—	—	—	—
1	High-straw	213 straw	0·65	1·55	112	34	0·39	0·38
2	Low-straw	111 starch + sugar 17 sawdust 87 straw	0·25	1·18	—	—	—	—
2	High-straw	248 straw	0·70	1·64	161	47	0·46	0·52

True digestibility of straw 100% with both animals.

III. *The metabolic faecal nitrogen excretion of the cow.*

Since, in order to determine the biological value of food proteins in the ruminant for maintenance or production, the cow or steer is the animal most generally used, it seemed advisable to obtain values for excretion of endogenous origin for the cow instead of assuming that the result for one species, the goat, would hold for other ruminants. For this purpose, two cows A and B were used, the low-N ration fed consisting of straw, maize and starch (Table VI).

Table VI.

	Starch equivalent lb.	N content g.
1 lb. flaked maize	0·84	7·25
12 lb. straw	2·52	17·40
2·25 lb. starch	2·25	1·02
200 ml. linseed oil	1·00	0·00
	6·61	25·67

Straw was used instead of paper or cellophane, since neither of these materials was eaten in sufficient amount to satisfy the needs of the animals for bulk in the ration. Since, however, straw had shown a remarkably high digestibility with goats, and since a further experiment with the two cows showed that the faecal excretion after ingestion of only 16 lb. straw did not differ from the excretion on the above diet, it seems safe to assume that the faecal N excreted has been very close to the actual metabolic faecal N. It can also be concluded that the maize had a digestibility very similar to that of straw. Further confirmation appears from the fact that the apparent digestibility of maize is over 90%, which will give a true digestibility of approximately 100%.

Preliminary periods of about 6 days were given with both animals before collection and analysis, while the actual experimental periods were 6 days for cow A and 9 days for cow B. A third experiment was performed on an animal (cow C) which had a rumen fistula. The straw was eaten voluntarily, while starch which formed the concentrate part of the ration was put into the rumen by way of the fistula. The cow was fed 2 lb. starch and 14 lb. straw. The results of all three experiments are shown below (Table VII). The food in all experiments contained 10% moisture.

Table VII.

Cow	Daily food intake lb.	Daily faecal N g.	Faecal N/100 g. dry food intake
A	15·5	29·9	0·48
B	15·5	26·0	0·42
C	14·0	25·0	0·44
		Mean	0·45

It appears that the faecal N of the cow like that of the goat and sheep is very high, the amount per 100 g. food intake being about double that of the one-stomached animal. The large quantity of indigestible carbohydrate in the diet of the ruminant may be the cause of this.

In order to find out the size of the constant fraction of the metabolic faecal N of the cow, cows A and B were starved for 7 and 9 days respectively (Table VIII). The faecal N of cow A became steady after 3 days and averaged 5·9 g. daily. The faecal N of cow B became steady after 2 days and averaged 10·1 g. daily. Two further cows C and D were starved for 9 and 12 days, while collections were made on the last 6 and 9 days respectively. The faecal N of cow C was 3·9 and of cow D 8·1 g. daily. If it had been possible to continue the starvation longer it is probable that the faecal N would have fallen still further, as in Carpenter's [1927] experiment on steers. During the period under observation, however, it remained fairly steady, and, since this period is one with a negligible food intake (the food which remained in the rumen when the fast commenced was slowly passed through the alimentary canal) it probably gives a fairly accurate figure for the constant fraction of the metabolic faecal N. It appears that the constant

Table VIII. *Daily faecal nitrogen excretion during starvation.*

Day of fast	Faecal N (g.)			
	Cow A	Cow B	Cow C	Cow D
1	25·91	26·67	32·00	20·48
2	17·51	15·50	22·84	9·56
3	14·64	15·14	6·68	22·36
4	6·39	10·50	3·98	5·93
5	5·12	11·80	4·25	9·13
6	4·93	5·16	5·51	9·78
7	6·53	10·61	2·07	8·03
8	—	8·86	2·50	7·50
9	—	12·95	4·84	8·96
10	—	6·46	—	8·72
11	—	—	—	3·65
12	—	—	—	11·59

fraction is specific for the different animals. The mean faecal N for all four cows during starvation was 7 g. daily, about one-quarter of the metabolic faecal N on a normal ration.

THE ENDOGENOUS URINARY NITROGEN.

During the above experiments a detailed study of the endogenous urinary excretion was also undertaken, together with a study of the fasting catabolism.

I. *The fasting catabolism of ruminants.*

Much work on fasting protein catabolism has been done in the past using one-stomached animals, experiments which, up to 1920, have been admirably reviewed by Cathcart [1920]. After that date little of note was produced until the publication of Boy [1934]. In a series of experiments, using rabbits, pigs and dogs as experimental animals, Boy claimed that the increase in N excretion during a fast following a N-free diet was composed in the main of urea, ammonia and creatine. The results, however, do not warrant the conclusions drawn, since whereas in some cases an increase in ammonia excretion was definite, in others no increase or even a decrease was found. Boy concluded that N metabolism in starvation is merely enhanced protein catabolism, similar to that found after ingestion of protein. It will be shown that the metabolism during a fast is very similar with all species of animals, both ruminant and one-stomached, and the conclusion that this can be reproduced by the feeding of protein appears to be correct.

In the past little attention has been paid to the N metabolism of the fasting ruminant. Grouven [1864] noted a daily loss of 50–60 g. urinary N in the cow. Various workers have determined the amounts of one or two constituents in the urine of fasting ruminants [Prayon, 1910; Palladin, 1924; Sjollema & van der Zande, 1923], but little of any importance was published until the detailed study with steers by Benedict & Ritzman [1927]. The full results of this investigation published by Carpenter [1927] showed that the N excretion per kg. body weight in 24 hours was 0·064–0·075 g. at a maximum. Expressed as percentages of the total N the urea N rose markedly whilst the amino-acid and hippuric acid N fell, the ammonia N remaining more or less unchanged. No ketonuria was noted, a fact which agreed with the findings of Sjollema & van der Zande [1923]. From the results Carpenter concludes that the effect of the previous diet had not disappeared till the 4th to the 6th day of the fast, from which day the true fasting base line can be taken.

Table IX. *Fasting catabolism of ruminants.*

Day of fast	Total N g.	Urea N g.	Ammonia N g.	Amino-acid N mg.	Preformed creatinine g.	Creatine g.	Sulphur g.	S : N
			Cow A. 417 kg. Low-N diet.					
1	14·95	4·31	5·90	0	3·97	0·74	2·31	1 : 6·5
2	19·94	7·21	4·94	870	5·40	1·80	2·01	1 : 10·0
3	19·16	8·61	2·47	110	3·16	3·44	1·08	1 : 17·7
4	28·13	16·75	2·15	140	5·06	1·59	1·20	1 : 23·4
5	27·32	17·77	1·83	40	3·68	1·92	1·15	1 : 23·7
6	49·45	29·24	2·14	270	5·52	3·18	2·16	1 : 22·9
7	54·12	32·36	2·69	370	5·65	3·09	2·24	1 : 24·1
			Cow. B. 453 kg. Low-N diet.					
1	19·71	4·19	5·17	320	4·73	0·25	1·26	1 : 15·7
2	34·06	20·68	2·12	250	6·39	0·90	2·52	1 : 13·5
3	42·67	28·50	2·10	260	5·95	1·78	3·06	1 : 13·8
4	41·88	28·70	1·40	240	5·45	1·92	2·88	1 : 14·6
5	34·98	21·94	2·42	150	5·99	1·60	1·13	1 : 30·9
6	32·21	19·13	2·47	340	6·12	1·90	5·04	1 : 6·4
7	40·76	24·58	3·95	300	6·52	2·03	2·47	1 : 16·5
8	46·71	26·07	7·48	340	6·23	2·40	3·42	1 : 13·7
9	43·96	27·43	4·57	150	5·69	1·72	2·59	1 : 17·0
10	42·78	23·05	5·55	440	5·31	1·24	2·58	1 : 16·6
			Sheep A. 37 kg. pasture.					
			mg.		mg.	mg.		
1	7·50	—	—	—	—	—	—	—
2	5·36	4·49	130	20	330	150	—	—
3	7·35	5·88	90	30	540	250	—	—
4	10·85	8·46	120	20	750	130	—	—
5	6·28	5·11	120	10	—	—	—	—
6	5·15	—	—	—	—	—	—	—
			Sheep B. 36·5 kg. pasture.					
1	—*	—	—	—	—	—	—	—
2	—†	—	—	—	—	—	—	—
3	15·22	12·20	300	140	1190	750	—	—
4	8·94	7·51	310	20	650	760	—	—
5	4·06	3·15	150	0	—	—	—	
6	5·63	—	—	—	—	—	—	—
7	7·16	6·28	480	30	—	—	—	—
			Goat 1. 62 kg. Low-N diet.					
1	5·07	2·72	1000	30	740	140	—	—
2	7·31	5·47	410	40	760	490	—	—
3	7·30	5·31	120	50	710	200	—	—
4	7·35	6·31	200	20	710	630	—	—
5	7·02	5·55	330	—	—	—	—	—
6	7·20	—	—	—	—	—	—	—
7	5·38	3·33	1040	40	—	—	—	—
			Goat 2. 24 kg. Low-N diet.					
1	2·05	0·95	40	10	390	0	—	—
2	3·76	2·69	90	30	520	60	—	—
3	3·67	2·84	120	40	390	70	—	—
4	1·73	1·29	80	20	220	150	—	—
5	1·15	—	150	—	—	—	—	—
6	4·58	—	—	—	—	—	—	—
			Goat 3. 40 kg. Low-N diet.					
1	2·83	2·08	190	20	—	—	—	—
2	5·56	2·88	160	40	1060	170	—	—
3	6·25	4·33	270	60	510	90	—	—
4	4·47	3·26	220	30	670	360	—	—
5	7·62	5·19	340	—	—	—	—	—
6	5·05	—	—	—	—	—	—	—
7	4·36	2·18	210	40	—	—	—	—

* First day of fast.

† First day of fast. No urination on first two days of fast.

In the present investigation the results are shown of the fasting catabolism of goats, sheep and cows following a low-N diet. It will be seen (Table IX) that starvation following a low-N diet causes an increase in urinary N excretion. With cow A the base line has been reached on the 6th day of the fast, whereas with cow B the level was reached on the 3rd day, although the previous diet was the same. With the goats and sheep the same marked rise in the output was noted. The daily N excretion per kg. body weight was 0·088–0·13 for the cows, 0·13 for the goats and 0·16 for the sheep.

Regarding the urinary partition, the most marked effects are to be seen in the increases in urea and creatine excretion. If, as Boy states, the fasting catabolism is merely an accelerated protein catabolism, the difference between the true endogenous level and the fasting level should be typical of protein metabolism. The sole difference between the two types of catabolism, endogenous and fasting, lies in the urea and creatine increases (Table X). A comparison of the differences between low and high protein diets shows that there is perfect agreement between the two types of metabolism, that of tissue protein and that of ingested protein.

Table X.

Period	Total N g.	Urea N g.	Ammonia N g.	Amino-acid N g.	Preformed creatinine g.	Creatine g.
			Goat 1.			
Low-N	2·52	1·19	0·47	0·059	0·28	0·01
Starvation	6·93	5·24	0·42	0·035	0·26	0·16
Difference	4·41	4·05	−0·05	−0·024	−0·02	0·15
			Goat 2.			
Low-N	1·40	0·56	0·22	0·038	0·18	0·02
Starvation	3·44	2·55	0·11	0·031	0·16	0·04
Difference	2·04	1·99	0·11	−0·007	−0·02	0·02
			Goat 3.			
Low-N	1·66	0·70	0·12	0·045	0·33	0·02
Starvation	5·55	3·63	0·24	0·044	0·29	0·08
Difference	3·89	2·93	0·12	−0·001	−0·04	0·06
			Cow A.			
Low-N	12·70	3·60	2·67	0·39	1·03	0·17
Starvation	39·70	24·00	2·20	0·20	1·33	0·65
Difference	27·00	20·40	−0·47	−0·19	0·30	0·48
			Cow B.			
Low-N	20·90	6·80	4·12	0·63	1·92	0·33
Starvation	40·60	24·40	4·22	0·28	1·60	0·49
Difference	19·70	17·60	0·10	−0 35	−0·32	0·16

Much work has been done on the sulphur excretion of the one-stomached animal during starvation. This has been collected and reviewed by Kahn & Goodridge [1926]. Discussing the N : S ratio in the urine, the authors state the old view that it is the result of catabolism of muscular tissue. The results do not appear to justify this statement completely, since the N : S ratio of muscle is about 16, whilst the N : S ratio of the urine during starvation is about 14. Terroine & Razafimahery [1935] have recently investigated the subject of S catabolism and have divided total starvation, following protein starvation, into three periods: (*a*) the first, in which a very marked but transitory increase in the N : S ratio occurs followed by as marked a fall—this is stated to be the result of partial catabolism of tissue proteins; (*b*) the second period, one in which the N : S ratio slowly decreases; a period in which, according to Terroine, all the tissues are being catabolized; (*c*) the third and premortal period, which shows marked increases in the output of urinary N and S, but a decrease in the N : S ratio of the urine.

With the ruminant the only work of note on S excretion during starvation is that of Carpenter [1927]. He found a constant increase in the N : S ratio as the fast progressed and in some cases obtained a figure of 60.

In the present experiment cow A showed a progressive increase in the N : S ratio, from 6·5 to 24·1, whereas cow B, except for days 5 and 6, showed a steady ratio throughout the experimental period. The significance of these results cannot be stated, but it is interesting to note that when the endogenous N and S outputs are subtracted from the starvation levels, the N : S ratio of the difference is very close to that of muscle tissue.

At present it is impossible to compare either these results or those obtained by Carpenter with Terroine's, since the period corresponding to Terroine's first period may be very much longer in the cow, owing to the large size of the animal. It seems essential that a fast of much longer duration be carried out with the cow to determine the exact significance of the S excretion.

It is worthy of note that only with two of the goats was any ketonuria noted, and this only intermittently and to a very small degree. This, as in the experiments reported by Carpenter and Sjollema, would presuppose complete metabolism of the body fat.

The undetermined N was high, as Carpenter also noted, but tended to increase in total amount as the fast progressed, although the percentage amount decreased markedly. It may be assumed with safety that this fraction consists of the purine bodies, and it appears that the ruminant tends to retain these compounds by minimizing cellular catabolism during a fast.

From a survey of all the results it seems possible to conclude that the N catabolism occurring during starvation is, for the first 7–10 days, the result of an enhanced protein breakdown.

II. *The nature of endogenous nitrogen metabolism.*

Much work has been done on the nature of the nitrogenous material catabolized during a N-free regime, since Folin [1905] postulated exogenous and endogenous forms of protein catabolism. Three theories regarding endogenous catabolism have been brought forward. Sherman [1920], confirming the earlier experiments of Osborne and Mendel [1914], assumed that the effect of ingested protein was to retard endogenous catabolism. When the ingested protein was completely efficient (i.e. as regards essential amino-acids) the endogenous N excretion would be minimum. This theory is completely opposed to the idea of a constant endogenous catabolism. Mitchell [1924] could not confirm the results of Sherman, finding, on the contrary, that all his evidence tended towards the theory of a constant endogenous catabolism. After reviewing all the work on the "minimum" endogenous catabolism Mitchell [1930] concluded that the precursors of the metabolites in all urine were the non-protein N compounds of the tissues, "each of which probably has some specific function to perform and is catabolized at varying rates in response to variations in the function it serves".

Terroine [1933] has reviewed the previous work and has concluded that the endogenous N metabolism arises from two sources. The urinary metabolites urea, ammonia and amino-acids, he states, arise from body protein catabolism, whilst the creatinine, creatine, allantoin and purine bodies arise from cellular catabolism.

Recently Roche [1934] analysed the tissues of rats dying from total starvation and from protein starvation and claimed that the water-soluble (extractable) N was an intermediary stage in tissue anabolism or catabolism. From the decreases in lysine, tyrosine and tryptophan after protein starvation, Roche concluded that with a N-free diet there is a progressive partial breakdown of muscle protoplasm. In other words, the endogenous N is protein in origin, a theory closely allied to that of Terroine.

With ruminants various experiments have been carried out to determine the endogenous N excretion [Morgen *et al.*, 1911, 1914; Hart *et al.*, 1912; Steenbock *et al.*, 1915; Honcamp *et al.*, 1923; Scheunert *et al.*, 1922]. For the cow values ranging from 0·029 to 0·045 g. N per kg. body weight have been obtained whilst with sheep the values range from 0·024 to 0·072 g. N.

In the present experiments urine was collected daily from sheep and goats on the N-free diets and from cows on the low-N diet. In Fig. 2 the total urinary N per kg. body weight is plotted against the food intake expressed as energy per kg. body weight. For the sheep the mean value per kg. body weight is 0·063 on the low food (energy) intake diet, for the goats 0·044 on the low and high food (energy) intake diets, and for the cows 0·039 on the low-N diet.

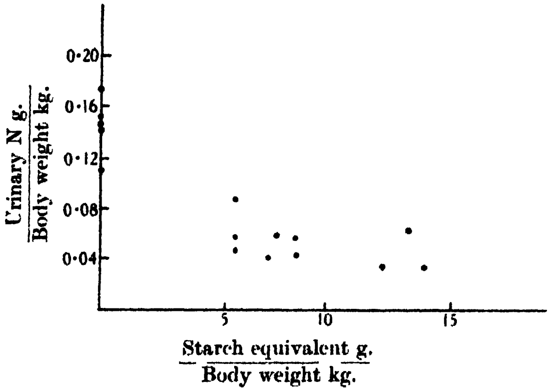

Fig. 2. Urinary N excretion of goats and sheep.

The urinary N partitions are shown in Tables X and XI. With all the animals as the food intake increases from the starvation level the percentages of urea and creatine decrease whereas the percentages of all the other constituents increase. From the results with the goats on the low and high energy intakes it appears that the true endogenous level of excretion has been attained on the low energy intake, except in the case of the creatine excretion, which is still slightly above the high energy level. With the cows the percentage urea excretion is slightly lower than with the goats and sheep, whilst the ammonia excretion is higher. Otherwise, with the three types of animals, the partition products expressed as a percentage of the total N output are very similar.

From a survey of the results it is impossible to state from what source the endogenous N has arisen, but experiments are in process to investigate this subject more fully.

SUMMARY.

1. Experiments were performed on goats, sheep and cows to obtain a figure for the endogenous excretion of N on a low-N diet.

2. The metabolic N excretion on a normal diet approximated 0·45 g. per 100 g. dry matter intake.

Table XI. *Partition products expressed as percentage of total N.*

		Urea N	Ammonia N	Amino-acid N	Preformed creatinine N	Creatine N
			Starvation.			
Goat	1	75·6	6·1	0·5	3·7	2·3
	2	74·2	3·2	0·9	4·6	1·2
	3	65·5	4·4	0·8	5·2	1·4
					% creatine + creatinine N of total N	
Sheep	3	80·2	1·6	0·2	3·36	
	4	82·5	3·1	0·3	5·50	
Cow	A	60·3	5·6	0·5	4·7	2·3
	B	60·0	10·4	0·7	5·5	1·7
			Low food intake diet.			
Goat	1	47·0	18·7	2·34	11·3	0·5
	2	40·2	15·8	2·70	12·5	1·5
	3	42·4	7·1	2·70	19·6	1·2
Sheep	1	51·0	11·2	1·70	13·0	2·6
	2	58·9	18·3	1·9	19·2	0·0
	4	70·2	11·9	1·0	11·2	0·1
			High food intake diet.			
Goat	1	47·1	11·0	1·7	11·1	0·4
	2	29·1	11·4	3·8	20·9	0·0
	3	39·2	16·8	1·5	20·5	0·0
			Low-N diet.			
Cow	A	26·9	19·9	2·9	11·4	1·8
	B	32·1	19·4	3·0	11·3	0·6

3. The effect of fibre on the N excretion was studied with results closely approaching those with the one-stomached animal, the greater the amount of indigestible dry matter the larger the faecal N. When this factor is taken into account it has been shown that the N of straw, which has an apparent digestibility of 30 %, has a true digestibility of 100 %.

4. A study of the constant fraction, the N excretion during starvation, has been undertaken.

5. The urinary excretion of N during fasting and on a low-N diet has been examined. During the fast the N excretion per kg. body weight varied from 0·088 to 0·13 g. with the cows, and to 0·16 g. with sheep. Further evidence that the fasting catabolism is merely an accelerated protein catabolism has been obtained.

6. The endogenous N excretion per kg. body weight varied from 0·063 with sheep, 0·044 with goats, to 0·039 with cows.

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CCXXXIX. THE DIGESTIBILITY OF DIETARY PROTEIN IN THE RUMINANT.

II. THE DIGESTIBILITY OF PROTEIN FOLLOWING A PROLONGED FAST, WITH A DETAILED STUDY OF THE NITROGEN METABOLISM.

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In the preceding paper [Hutchinson & Morris, 1936] it was shown that the faecal N could be divided into three fractions, (*a*) a constant fraction, (*b*) the true metabolic faecal N and (*c*) a fraction dependent on the N content of the diet. Only the first and second fractions were dealt with. The present publication deals specifically with the third fraction.

In a series of publications on the nutritive value of proteins for milk production Morris & Wright [1933, 1934] noted that, when foods containing protein of high biological value were ingested, a lowered N digestibility was found. This general relationship is shown in Fig. 1. Further work has confirmed the results [Morris & Wright, 1936]. Moreover, in another series of experiments designed to determine the nutritive value of proteins for maintenance [Morris & Wright, 1935] a similar general relationship was obtained. When this observation was first noted the authors wrote: "At first sight it seemed possible that this might be due to normal variations in digestibility..., but the possibility must be considered that the body itself can to some extent adjust the assimilation of N from, or re-excretion of N into the gut according to its needs....It is not unreasonable to assume that absorption from the gut might be stimulated when inadequate rations were fed." They also suggested an investigation into the effect of varying levels of food intake on digestibility.

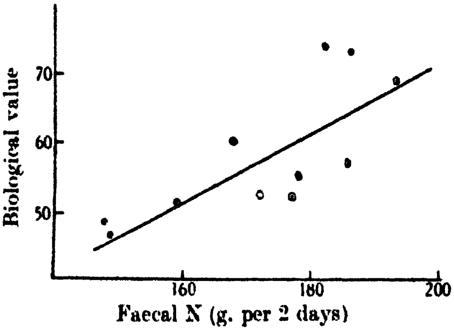

Fig. 1. Relation between biological value and faecal nitrogen.

The immediate object of the present series of experiments was to determine whether the same food would show an altered apparent digestibility when fed at the same level to an animal under circumstances in which the requirements of that animal for essential amino-acids varied markedly.[1] This was carried out by feeding a low-N diet to an animal and following this with a period of prolonged starvation until the animal was in marked N debt. When a large amount of N had been lost, a protein of high biological value was added to the basal diet. This ration was continued until the N deficit had been reduced to minimum amounts. Any alteration in the faecal N excretion could then at once be observed.

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[1] In previous work it has been shown that the biological value of proteins for ruminants depends chiefly on their contents of lysine and tryptophan.

Experimental method.

Two cows were used, both having terminated their period of lactation about 30 days before the commencement of the experiment. The rations fed are shown in Table I. Bean meal was selected as the protein of high biological value since it has been shown [Morris & Wright, 1933] that for milk production it has a fairly high value, about 60, while Morris [1934] has shown that the % N as lysine and tryptophan is relatively high. The basal ration of straw and maize remained constant throughout the feeding periods, but the starch intake was varied with the addition of the beans in order to keep the energy content of the diet steady throughout the complete experiment. A final period in which gelatin replaced the bean meal was included.

Table I. *Details of daily rations for both animals.*

Period	Ration	N intake g.	Energy intake as lb. starch equivalent
1	1 lb. maize 12 lb. straw 2·25 lb. starch 200 ml. linseed oil	25·67	6·61
2	Starvation	—	—
3	1 lb. maize 12 lb. straw 3·75 lb. bean meal 200 ml. linseed oil	92·65	6·84
4	1 lb. maize 12 lb. straw 1·25 lb. bean meal 1·65 lb. starch 200 ml. linseed oil	47·32	6·84
5	Same as period 3	92·65	6·84
6	1 lb. maize 12 lb. straw 1 lb. gelatin 0·5 lb. starch 400 ml. linseed oil	92·65	6·80

DISCUSSION OF RESULTS.

(a) *Nitrogen balance.*

The N balances for each period are shown in Table II. It will be seen that before feeding the first high protein diet the two animals were in N debt to the extent of 394 g. and 697 g. respectively. At the commencement of the second period of high protein the debts were only 75 g. and 282 g. N. The former is minimum and can be ignored, but the latter is definitely high. It may be noted that, whereas cow 1 had a fairly large positive balance on the second period of low-N diet, cow 2 registered a loss of N. This is peculiar since cow 2 was in N debt to a far greater extent during this period of low-N diet than cow 1. From the balance figures it is obvious, however, that, in the first period of high protein, requirements were high, whereas in the second period for cow 1 they were minimum. With cow 2 the N requirements were minimum about midway through the second period of high protein feeding.

Table II. *Nitrogen balances.*

Period	Intake g.	Output g.	Balance g.	Total loss g. N	Total loss g. N
		Cow 1.			
1. Low-N	154·02	254·30	−100·28	394·37	75·07
2. Starvation	0·00	294·09	−294·09		
3. 1st high-N	926·50	670·51	+255·99		
4. Low N	851·76	788·45	+ 63·31		
5. 2nd high-N	741·20	482·78	+258·42	—	—
6. Gelatin	1019·15	787·73	+231·42	—	—
		Cow 2.			
1. Low-N	231·03	424·73	−193·70	697·26	282·08
2. Starvation	0·00	503·56	−503·56		
3. 1st high-N	1667·70	1225·39	+442·31		
4. Low-N	615·16	642·09	− 27·13		
5. 2nd high-N	1111·80	816·71	+295·09	—	—
6. Gelatin	370·60	274·04	+ 96·56	—	—

(*b*) *Faecal nitrogen excretion.*

Daily faecal N excretions are shown in Fig. 2, and the corresponding figures for the digestibility during each period are shown in Table III. Following the very low output during starvation, there is a rise in faecal N in the first period of high protein feeding. The level is, however, not significantly higher than that found during the ingestion of the low-N diet, and the digestibility of bean meal is 90%. With cow 1 the period on low-N can be subdivided into two portions. The first is that in which bean meal shows a digestibility of 100% and the second where the faecal N rises markedly and the digestibility falls to about 73%. With cow 2 the digestibility falls immediately to about 70% in the low-N

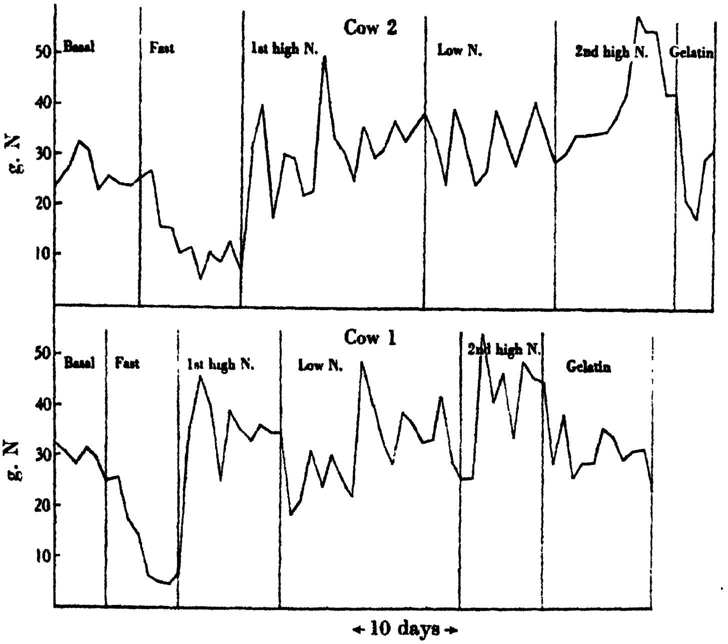

Fig. 2. Faecal excretion.

Table III. *Digestibilities of bean meal and gelatin.*

Period	Intake of N added to basal diet g.	Output of faecal N g.	Basal output of faecal N g.	% digestibility
		Cow 1.		
1st high-N	66·98	36·34	29·68	90·0
Low-N (*a*)	21·65	24·85	29·68	100·0
Low-N (*b*)	21·65	35·58	29·68	72·8
2nd high-N	66·98	43·07	29·68	80·0
Gelatin	66·98	30·36	29·68	99·0
		Cow 2.		
1st high-N	66·98	32·00	26·29	91·5
Low-N	21·65	32·50	26·29	70·1
2nd high-N (*a*)	66·98	34·77	26·29	87·3
2nd high-N (*b*)	66·98	49·64	26·29	65·1
Gelatin	66·98	25·55	26·29	100·0

period. When the high protein diet is again resumed the digestibility of the bean meal shows a figure of 80% with cow 1, whereas the decrease below the level of the first high-N period only occurs midway through the period with cow 2. With gelatin the digestibility rises once more to 100%.

The total weight of faeces and the percentage dry matter are of interest (Table IV). Comparing Tables III and IV it will be seen that an increase in faecal N corresponds to an increase in total faecal weight, with a very slight increase, if any, in the amount of dry matter excreted. The faecal N varies directly with the moisture content of the faeces, the digestibility of total dry matter remaining constant. This fact was noted by Titus [1927]. It appears that the apparent digestibility of any foodstuff is not related to the digestibility of the total dry matter ingested, but varies rather with the moisture content of the faeces.

Table IV. *Amount of faeces and dry matter excreted.*

Period	Wt. of faeces (wet) g.	Wt. of faeces (dry) g.	Dry matter %
	Cow 1.		
1st high-N	15,475	3021	19·5
Low-N (*a*)	13,286	2222	16·7
Low-N (*b*)	18,591	2528	13·6
2nd high-N	20,630	2569	12·4
Gelatin	14,357	2170	15·1
	Cow 2.		
1st high-N	11,677	2118	18·1
Low-N	14,150	2381	16·8
2nd high-N (*a*)	16,125	2387	14·8
2nd high-N (*b*)	20,583	2855	13·9
Gelatin	12,688	2005	15·8

The low digestibility of the N given in the low protein period in cow 2 is presumably due to a temporary irregularity, since the water content of the faeces in this period was not high, nor was the faecal weight. The error of the experiment is naturally larger when a low-N diet is fed.

(*c*) *Nature of the faecal nitrogen.*

Having obtained evidence that with the same food different apparent digestibilities could be observed according to the requirements of the animal, it seemed desirable to investigate the nature of the excess faecal N. Dried samples of the faeces which had been collected throughout the experiment were therefore analysed for total N before a more detailed examination. The results were unexpected, for the whole of the excess N was found to be lost on drying at 180° for 1 hour. The samples of faeces obtained during the first high-N, the second high-N and the gelatin periods were analysed. The results are summarized in Table V, the mean percentage loss on drying being recorded side by side with the percentage digestibility. It will be seen that as the digestibility falls the percentage loss rises. Considerable variations were found between individual samples, as is apparent from the relatively high standard deviations. The differences between the first and second periods are not statistically significant, between the second and third periods they are barely significant, but between the first and third periods they are definitely significant—the probability of such differences occurring by chance being less than 1 in 100.

Table V. *Loss of nitrogen on drying faeces at 180° for 1 hour.*

Period	% digestibility	No. of samples analysed	Mean % loss on drying	Standard deviation
		Cow 1.		
Gelatin	99·0	6	2·86	7·09
1st high-N	90·0	10	9·60	7·75
2nd high-N	80·0	8	25·97	9·34
		Cow 2.		
1st high-N	91·5	8	2·52	9·27
2nd high-N (*a*)	87·3	5	7·94	7·40
2nd high-N (*b*)	65·1	4	19·22	4·03

From these facts it seems safe to conclude that the excess N excreted during the later periods of high-N feeding is composed of compounds which are (*a*) volatile or decomposed at 180°, or (*b*) transformed by heating at this temperature into nitrogenous bodies which cannot be estimated by the Kjeldahl method, as, for example, compounds containing the pyrrole ring structure.

(*d*) *Total urinary nitrogen.*

No detailed study of the changes in N metabolism following prolonged periods of both protein and total starvation has been undertaken in the past. Benedict & Ritzman [1927] make a brief reference to one or two results with starving steers. They found that for the first two days after feeding there was a marked fall in total N excretion together with a fall in both total and percentage urea and creatine N. The amino-acid N and hippuric acid N, on the other hand, increased markedly, whilst only a very slight percentage increase in ammonia N was noted. These changes were particularly noticeable after the first period of re-feeding. In the present experiment a detailed study of re-alimentation in the ruminant is presented.

Fig. 3 shows the urinary N throughout each feeding period, together with the results of the previous protein and total starvation periods. Two facts are evident from the results. The urinary N during the period of high protein feeding immediately following starvation is only slightly less than that found during starvation. As with Benedict's steers the first feed caused a marked fall in urinary N, with a rise in the succeeding days. Also the output in this period is higher than in the succeeding high protein period. Calculation of the percentage utilization of the absorbed N clearly illustrates this difference. With the first high-N diet the N excretions due to the food intake are 18·0 and 15·2 g. respectively for the two cows (obtained by subtracting the endogenous N of the period before the starvation from the average daily excretion). The total absorbed N is 86 g. daily, which gives utilizations of 80 and 83 %. In the second high-N period the nitrogen excretions were 4·7 g. and 4·6 g. respectively. With a true intake of 65 g. N daily there are utilizations of 93 % with both animals. There are two possible explanations of this result. Either the caloric requirements immediately following starvation may be higher than the normal requirements, resulting in the deamination of amino-acids for energy purposes, or the ability of the animal to retain absorbed N may be somewhat impaired following a prolonged period of undernutrition.

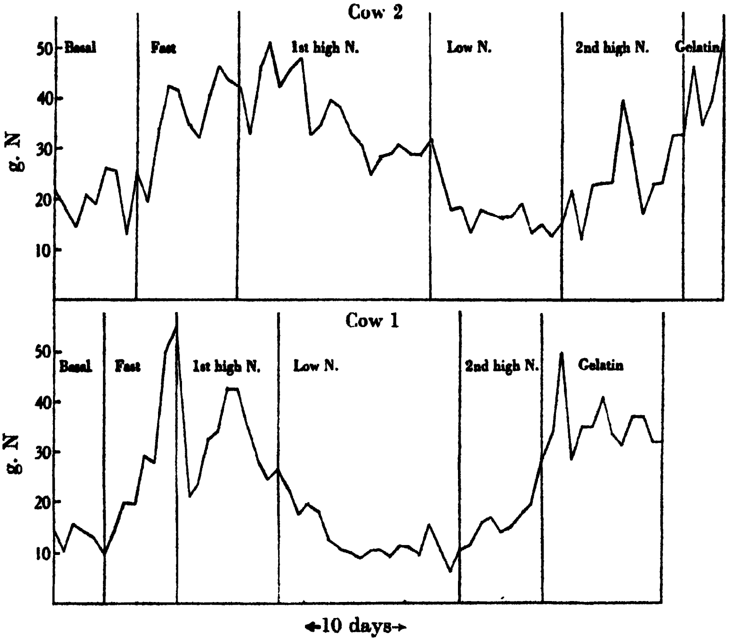

Fig. 3. Urinary excretion.

The second fact of importance is that the urinary excretion in the second low-N period is lower than that in the first, before the starvation. This is interesting since the N intake in the second period is 16 g. higher daily, being supplied in the form of bean meal, a protein which was found to have a fairly high biological value for milk production [Morris & Wright, 1933]. From the very low N intake during the first low-N diet and from the maintenance requirements of the cow [Morris & Wright, 1935] it has been assumed that the urinary N excretion during this period is endogenous N. Since the addition of a fairly large amount of bean meal protein causes a decrease in N excretion, it appears that there is some evidence, in the ruminant, for Sherman's [1920] theory of decreased endogenous N with addition to the diet of a protein of high biological value.

With gelatin, owing to deficiencies of certain essential amino-acids, the urinary N excretion is higher than with bean meal.

(e) *Urinary partition of nitrogen.*

From a study of the N partition in the urine (Table VI) it will be seen that ingestion of food after a period of starvation causes marked alterations. Whereas

Table VI. *Average daily urinary partition of nitrogen following starvation.*

Figures in brackets represent percentages of total N.

Ration	Total N g.	Urea N g.	Ammonia N g.	Amino-acid N g.	Preformed creatinine N g.	Creatine N g.
			Cow 1.			
Starvation	39·70	24·00 (60·3)	2·20 (5·6)	0·20 (0·5)	1·33 (4·7)	0·65 (2·3)
1st high-N	30·70	9·90 (32·2)	7·20 (23·4)	1·00 (3·3)	2·17 (7·1)	0·36 (3·6)
Low-N	10·41	1·36 (13·1)	2·57 (24·7)	0·42 (4·0)	1·63 (15·6)	0·18 (1·7)
2nd high-N	17·27	5·00 (28·9)	3·08 (17·8)	0·34 (2·0)	1·67 (9·7)	0·49 (2·8)
Gelatin	35·28	17·32 (49·1)	10·78 (30·5)	1·20 (3·4)	1·91 (5·4)	0·36 (3·2)
			Cow 2.			
Starvation	40·60	24·40 (60·0)	4·22 (10·4)	0·28 (0·7)	1·60 (5·5)	0·49 (1·7)
1st high-N	36·08	15·84 (43·8)	8·30 (23·0)	0·92 (2·6)	2·25 (6·2)	0·15 (0·4)
Low-N	16·86	4·64 (27·5)	3·44 (20·4)	0·36 (2·0)	2·01 (11·9)	0·10 (0·6)
2nd high-N	25·86	9·38 (36·3)	4·68 (18·0)	1·14 (4·4)	2·39 (9·2)	0·31 (1·2)
Gelatin	42·96	—	—	—	—	—

the percentage of urea N falls the percentages of the other products increase markedly. These results confirm the findings of Benedict & Ritzman [1927].

It is of interest to note the difference between the partitions on the low and high protein (2) diets when the protein requirements are high. An increase is found in both total and percentage urea N excretions, whereas little change can be seen in the ammonia and amino-acid excretions. The preformed creatinine excretion remains constant in amount although the percentage falls on changing to the high protein ration. The creatine output increases in both total and percentage amounts. These results, except for the ammonia N excretion, agree closely with the results obtained by Folin [1905] on man.

When, however, the ingested protein cannot be used for tissue production purposes, as in the first high protein period and the gelatin period, the partition products are altered. Marked increases in total urea, ammonia and amino-acid N are found. As regards percentage output, the urea N alone shows a marked increase, the ammonia and amino-acids remain fairly constant, while the creatinine decreases.

From the complete series of results it appears that, with the ruminant, increased deamination leads to an increase in the total amounts of urea, ammonia and amino-acid N excreted. With the creatine no definite conclusions can be drawn, and it may be concluded that deamination has little effect on the urinary excretion of creatine. On the other hand, when the ingested protein is being used for tissue anabolism, a definite increase in creatine output occurs. Similarly during tissue catabolism, as in the starvation results, a marked increase in creatine output is found. It is evident that creatine excretion in the ruminant is controlled by cellular activity and is in no way affected by that portion of the ingested protein which is deaminated either to supply energy needs or because an excess of protein has been ingested in the diet.

(*f*) *Sulphur excretion.*

It has been shown in the previous paper [Hutchinson and Morris, 1936] that during starvation the N : S ratio of the excreted products is, after deducting the endogenous excretion, the same as that of muscle tissue. In the present paper the effect of feeding following a fast is studied, i.e. the changes which occur in the S excretion when anabolism is in progress. Wilson [1933] has shown that, with the one-stomached animal, S excretion during tissue catabolism and S retention during anabolism precede N excretion and retention. During starvation (i.e.

tissue catabolism) S excretion appears to precede N excretion, the N : S ratios being less than that for body tissue.

With the ingestion of food the system is reversed, anabolism replacing catabolism, and retentions of N and S replacing losses. The effect on the N : S ratio is shown in Table VII.

Table VII. *The effect of a high protein diet following starvation on the daily N : S ratio in the urine.*

Daily N : S ratio.

Animal	Day 1	2	3	4	5	6	7	8	9
Cow 1	3·8	6·4	5·3	6·1	8·2	7·3	5·7	3·0	4·5
Cow 2	3·8	9·6	11·0	10·4	11·8	12·1	9·0	7·9	6·4...

The first feed causes a marked decrease in excretion of urinary N with little change in the S output, thereby giving a N : S ratio very much lower than that found during starvation. This is followed by an increase in the ratio, showing that S is being retained in advance of N. This period, lasting about 7–8 days, is then followed by one in which N retention increases, with a concomitant decrease in the N : S ratio in the urine. It is evident that, with the ruminant, as with the one-stomached animal, S metabolism precedes N metabolism.

The N and S intakes and outputs together with the N : S ratio of the material retained are shown in Table VIII. The results show clearly that short experiments

Table VIII. *Nitrogen and sulphur excretions.*

	True intake		Excreted		Retained		
Ration	Nitrogen g.	Sulphur g.	Urinary N g.	Urinary S g.	Nitrogen g.	Sulphur g.	N : S of retained material
			Cow 1.				
1st high-N	83·38	8·74	30·70	5·80	52·68	2·94	17·8
Low-N	31·55	4·78	10·41	4·20	21·14	0·58	36·4
2nd high-N	74·12	8·74	17·27	3·52	56·85	5·22	10·9
N : S ratio of total N and S retained in all three periods. Mean					43·56	2·91	15·0
			Cow 2.				
1st high-N	84·50	8·74	36·08	4·58	48·42	4·16	11·6
Low-N	35·50	4·78	16·86	4·60	18·64	0·18	103·6
2nd high-N	81·65	8·74	25·86	5·40	55·79	3·34	16·7
N : S ratio of total N and S retained in all three periods. Mean					40·95	2·56	16·0

with ruminants may lead to entirely wrong conclusions. It will be seen that with cow 1 the N : S ratio of the retained material in the first period of high protein, and in the period of the low protein ration, shows that N is retained over each complete period to a greater extent than S, whereas in the last period the position is reversed. With cow 2 the first high protein period shows a greater retention of S whilst the low protein ration gives an extremely high N : S ratio, the result of an intense retention of N. Taking the mean of all three periods, however, it will be noted that the retained material has a N : S ratio very close to that of body tissue. It is clear that, using the ruminant as the experimental animal, correct conclusions can only be drawn with long experiments.

(*g*) *Biological values.*

Much work has been done in the evaluation of proteins for the growth of the young, chiefly using the rat as experimental animal. So far there has been no record of any attempt to find the biological value of proteins for growth, weight increase or tissue repair *in adults*. The present series of experiments affords an opportunity to study this problem. During the periods on low N intake and starvation the animals suffered severe losses of body tissue, chiefly protein. This is readily seen from the large N deficit at the end of the fast. During the period of high protein intake and in the subsequent periods the retention of N was high. In those periods following the fast there is, therefore, opportunity for the study of the nutritive value of the proteins ingested. As in all studies of the biological value for production, it must be remembered that the results are for both production and maintenance, since no method has as yet been evolved which can separate these two functions.

The results for the proteins used in the present experiment, bean meal, straw and maize, are shown in Table IX. It will be noted that the results for the two animals are in remarkably close agreement. The fall in value between the low protein period and the second high protein period is due mainly to the decrease in apparent digestibility, giving an increase of 10 g. in the daily faecal N. The fairly high biological value of the gelatin-containing diet, i.e. 75, can be understood when it is remembered that although the gelatin is deficient in tryptophan the straw protein has 15 % tryptophan [Morris, 1934]. This will, in part, make up for the gelatin deficiency.

Table IX. *Biological values for tissue repair.*

$$\text{B.V.} = \frac{I - (F_t - F_m) - (U_t - U_e)}{I - (F_t - F_m)}.$$

Period	Protein level %	Intake	F_t	F_m	U_t	U_e	B.V.
			Cow 1.				
1st high-N	18	92·65	36·34	29·65	30·70	12·70	80
Low-N	7	47·50	31·40	29·65	12·40	12·70	100
2nd high-N	18	92·65	43·07	29·65	17·28	12·70	94
Gelatin	18	92·65	31·69	29·65	36·05	12·70	74
			Cow 2.				
1st high-N	18	92·65	32·00	26·25	36·08	20·91	82
Low-N	7	47·50	32·53	26·25	16·86	20·91	100
2nd high-N	18	92·65	42·06	26·25	24·42	20·91	95
Gelatin	18	92·65	25·55	26·25	42·96	20·91	76

I = intake. F_t = total faecal N. F_m = metabolic faecal N, obtained from the period of low N intake before starvation. U_t – total urinary N. U_e = endogenous urinary N obtained from period before starvation.

The most important point, however, is the fact that an 18 % protein level can give, for growth of tissue, a biological value of 95, very little less than the figure obtained with a 7 % level. Clearly this type of experiment would be of value in estimating the nutritive properties of any type of protein or non-protein compound for growth purposes.

It is of interest to compare the results obtained with ruminants, in which beans formed the major portion of the diet, with those obtained with the one-stomached animals. In the latter, beans have a fairly low biological value owing both to low digestibility and low content of cystine. With the ruminant this experiment affords further evidence that cystine, if essential for ruminant nutrition, is only essential in minimum amounts. This fact has already been shown for maintenance [Morris & Wright, 1935].

SUMMARY.

1. Experiments were performed on cows to discover the effect on N metabolism of re-alimentation following a prolonged fast.

2. The faecal N excretion shows that the apparent digestibility of a protein varies according to the requirements of the animal and also according to the biological value of the protein ingested. The higher the biological value of a protein the lower is the digestibility. Similarly, the greater the requirement of the animal for protein the higher is the digestibility.

3. The excess N excreted during those periods when the apparent digestibility was low was found to be lost on heating at a temperature of 180°.

4. The urinary N shows that, immediately following prolonged starvation, there is poor utilization of absorbed N, owing either to increased demand for energy, or to impaired ability to retain absorbed N.

5. N partition products in the urine show that during active tissue anabolism a marked increase in creatine output occurs, whereas no alteration takes place when the absorbed N is deaminated and excreted.

6. S excretion and the N : S ratio show that in tissue anabolism S is retained in advance of N.

7. Calculation of the biological value of the ingested protein showed that, following a prolonged fast, an 18 % protein level had a value for tissue growth very little less than a 7 % level.

The authors are indebted to Dr N. C. Wright and Dr H. Chick for their invaluable advice and criticism.

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CCXL. SPECTROGRAPHIC STUDIES ON THE ANTIMONY TRICHLORIDE REACTION FOR VITAMIN A.

I. THE RELATION BETWEEN TINTOMETER READINGS AND SPECTRAL ABSORPTION OF THE BLUE SOLUTION.

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According to our present knowledge there seems no doubt that there exists an intimate connexion between vitamin A and the blue colour produced with a number of dehydrating agents, especially antimony trichloride in chloroform. The colour follows the vitamin even into the most potent concentrates, approaching 100 % purity, and no really serious discrepancies are on record that cannot find a rational explanation. The reaction has gained a firm footing, both in science and commerce, on account of its simplicity, even though a number of sources of error adhere to it. Very much has been written about this reaction, but it appears to the present writers that several of the fundamental facts have not yet been fully explained.

No really satisfactory explanation has yet been found for the relation between concentration and blue value, as measured in the Rosenheim-Schuster Lovibond Tintometer, within the range of reasonably accurate measurement, i.e. B.V. about 4–12.

For potent oils and concentrates giving a blue value of 6 for concentrations of about 1 g./l. and lower most authors agree that the relation is linear, within the usual range of measurement [Norris & Church, 1929; 1930; Smith & Hazley, 1930]. But at least two of the graphs of Norris & Church contradict this, and a number of the straight lines of Smith & Hazley might equally well have been drawn as curves. Evers [1929], on the other hand, finds lower values the higher the quantity taken, especially of oils with a high vitamin content, e.g. ox liver fat.

Norris & Church [1930] show curves for a number of oils; they do not find proportionality even over a small range, and find no mathematical relation which satisfies all their curves. It seems that they exaggerate the accuracy of tintometer readings, according to our experience. Drummond & Hilditch [1930] allege that the colour intensity is a linear function up to "10 % concentration"; at higher concentrations this does not hold ("10 %" corresponds to 9·09 g./l.). The fact that several of the straight lines of these authors do not pass through the origin seems difficult to explain. Coward *et al.* [1931] say that it is a well-known fact that the relation between intensity of colour and concentration of an oil is not linear, but is represented by a line which falls away at higher concentrations to a curvilinear function. Gillam & Morton [1931] recommend some "caution in using the Lovibond technique as a quantitative method of assay...".

Notevarp & Hjorth-Hansen [1931] in a publication from this Institute state that the colour/concentration relation for oils follows an exponential function

$$B_c = b_1 . c^{0 \cdot 7}$$

where b = the colour obtained by the concentration 1, B = the colour obtained by the dilution c.

The relation has also been mentioned in another publication [Notevarp, 1935].

Apart from slight discrepancies in the accurate wave-lengths concerned, it is agreed that the colour given by potent oils and concentrates is due to absorption bands at 617–620 $m\mu$ and 580–585 $m\mu$, the former predominating in the relation of about 10:5 to 10:6.

In less potent oils, such as ordinary cod liver oil, the bands are displaced to 600–606 $m\mu$ and 570–575 $m\mu$ respectively. Their relative intensities may vary within wide limits, either may be the strongest although the 606 $m\mu$ band usually predominates.

The shift of the bands is probably due to the change of solvent effected by the oil; that bands shift when the solvent is changed is well known [v. e.g. Scheibe, 1925]. The other changes in the bands will be treated in another publication which we hope will be forthcoming shortly.

In this publication are given the wave-lengths found by means of an empirical scale on the instrument at our disposal, 572 $m\mu$ and 603 $m\mu$ for oils, 580 $m\mu$ and 618 $m\mu$ for concentrates. We do not consider the exact situation of the bands to be of major importance.

The qualities of the reagent, such as concentration, temperature, contents of moisture and impurities, age etc. are factors which influence the result. It has been shown by several authors that the intensity of blue increases with the concentration of antimony trichloride [Wokes & Willimott, 1927, 1, 2; Norris & Church, 1930; Brode & Magill, 1931, and others]. This has been confirmed in detail at the Fisheries Research Station; it is hoped that it will be possible to publish these results later, and it should only be mentioned here that the colour/concentration curve appears to approach asymptotically to a maximum value. This cannot actually be reached, but this means that small variations in reagent concentration are insignificant when the solution is nearly saturated. For practical application, however, 220 g./l. is now universally accepted as the standard concentration, so this question need not be discussed here. The same applies to temperature, 18° being now universally employed. Water and alcohol, impurities both likely to occur, lower the results; they should be entirely removed from the chloroform and kept away from the reagent. Light alters the reagent in the course of time, probably by the formation of decomposition products of the solvent. This indicates the necessity for avoiding unnecessary exposure of the chloroform to light and air during purification, otherwise the result may be a reagent which does not give the maximum values from the outset.

EXPERIMENTAL.

All reactions with antimony trichloride reagent have been carried out at 18° ± 0·5°; reagent concentration 220–225 g./l. throughout. Every care has been taken in preparing the reagent; the chloroform has been washed, dried with freshly heated potassium carbonate and distilled over a small amount of calcium hydride. For the last batch the distillation was carried out in a partial vacuum, at about 30°, as difficulties were encountered in getting the reagent to give maximum values. A whole sample of trichloride was taken for each lot of reagent, and poured straight from the newly-opened bottle into the chloroform. After dissolving by warming to about 25°, the concentration was determined, chloroform added if necessary, and the reagent transferred to the stock bottle by sucking it over into the evacuated bottle through a U-bent glass tube with a stopcock. In this way contact with moist air was reduced to a minimum, and sediments in the solution were left in the first flask.

The reagent was measured out by means of a very simple burette designed first by the late R. Engström, the design being improved by one of the authors (H. W. W.) to that shown in Fig. 1. The burette is filled either by compressing the air in the bottle or by suction through the top. Ordinary corks have been used, they are fitted in the empty apparatus after softening with steam and dried in place. The burette is calibrated by comparing the weight of reagent measured out with the weight of the contents of an ordinary pipette. This is necessary because the tip below the cock, which should be as small as possible, retains an amount which differs for fluids of varying viscosity and capillarity.

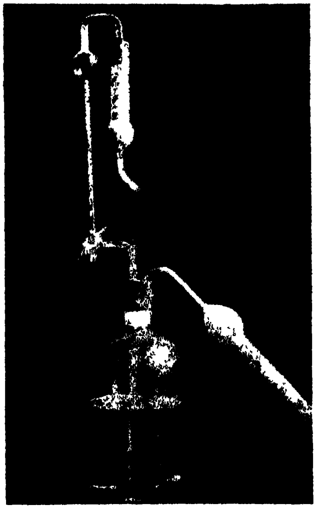
Fig. 1.

The oil solution was run into the cell from a 1 ml. pipette graduated to 0·01 ml. and the reagent added. Immediately afterwards the mixture was stirred by a current of dry air blown in through a thin glass tube with a capillary opening. This gives a very satisfactory stirring. The amount of chloroform vapour carried off reduces the volume less than 0·2 %. Nitrogen was first employed, but it was found later that the oxygen in the air does not affect the reaction to any noticeable extent. Apparently the reaction is much less sensitive to disturbances after the reagent has been added.

Oils and concentrates were diluted for the antimony trichloride reaction with the same chloroform as that used for making the reagent, after this had been evacuated to remove dissolved air and saturated with CO_2. The solutions were examined within a few hours of preparation, most of them within an hour.

A tintometer of the British Drug Houses' design has been used for the determination of blue values.

Spectrographic measurements have been carried out on a Zeiss "Spektrograf fur Chemiker" by the rotating sector method, with photographic recording. For the yellow and orange bands several panchromatic materials have been found satisfactory. The best material we have found are "Agfa Rot Hart" plates. Where possible 5 sec. have been used as standard time of exposure, varying the aperture so that the product of this and the sector opening is constant.

All the oils examined have been of wholly known origin, either produced by the Fisheries Research Station or received from the producer direct. What is said applies therefore only to pure cod liver oil, which has undergone no refining or other process which might alter its chemical or physical properties. The concentrates have been prepared with every possible care in the accepted way.

Expression of results. All concentrations are expressed in accordance with the Paris Convention for the Expression of Analysis of Foodstuffs.

Concentrations in connexion with the antimony trichloride reaction are expressed as g./l. or mg./l. chromogenic substance in the reaction mixture. The small variation in $SbCl_3$-concentration resulting from using varying amounts of chromogen solution (0·05 to 0·5 ml.) does not appreciably influence the results. Concentrations of concentrate solutions are expressed as the corresponding concentrations of the oil, to make comparison possible.

Intensity of spectral absorption is expressed by the extinction coefficient E for a 10 g./l. solution, as defined by Bunsen & Roscoe, see International Critical Tables. This, by the way, does not contain the unnecessary reference to a 1 cm. cell so frequently met in publications on spectrography.

RESULTS.

Change in absorption with time.

The blue colour changes with the time after the reaction is started; the absorption maximum changes and the shape of the absorption curve is altered. To be certain of observing the maximum of absorption, and also to study the shape of the curve, a number of observations were carried out at intervals after mixing on almost every blue solution measured.

The equipment allows the first exposure to begin 5–7 sec. after the reagent has been added. For concentrates it appears that the maximum is reached at or before this time; exposures from 20 to 25 sec. already show an appreciable fading. The 618 $m\mu$ band is always the strongest except in the case of partially destroyed concentrates. Both bands rise and fall simultaneously.

The alteration with time of the absorption curve for oils varies. Normal oils, with the 603 $m\mu$ band predominating, give the 572 $m\mu$ band maximum, masked by the rising 603 $m\mu$ band; almost immediately this band then fades. The 603 $m\mu$ band reaches its maximum after about 30 sec., remains nearly constant for about 25 sec. and then fades slowly (Fig. 2). In the tintometer this will be seen by the colour being at first of a purple hue, turning gradually more greenish. As the complementary colour of 572 $m\mu$ is a colour at the beginning of the purple line, whereas the complementary of 603 $m\mu$ is at 495 $m\mu$, an aquamarine colour, this is easily explained.

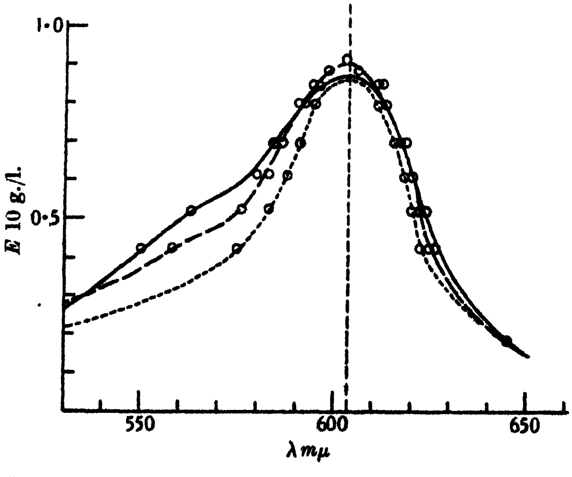

Fig. 2. —— 10–15 sec.; - - - 35–40 sec.; · · · · 85–90 sec.

The opposite is the case with oils with the greatest absorption at 572 $m\mu$. Here the 603 $m\mu$ band reaches its maximum in 10 sec. or less and fades very quickly, whilst the 572 mμ band develops its maximum in the course of 60–90 sec. and remains stable for a comparatively long time. In the tintometer this is seen as a very transient aquamarine colour, changing to a more and more purplish blue. In the case of very high oil concentrations the intensity of the 603 mμ band is greatly reduced, and the development of the 572 band is delayed: it also becomes narrower, the maximum, however, being the same. The concentrations in question are far above (2–3 times) those in practical use.

The curves of Fig. 2 have been drawn so that the marking decreases with time, to attempt to give a stereoscopic effect. Intermediate values have been omitted for clarity; they do not contribute anything particularly noteworthy.

The antimony trichloride reaction and Beer's law.

The results published by Brode & Magill [1931] indicate that the absorption is proportional to the oil concentration. On the other hand, a number of authors allege that the oil inhibits the reaction in higher concentrations. We have therefore measured a number of oils, especially weak oils in high concentrations, to find out whether such inhibition takes place. We have also measured more normal oils and concentrates, although if the weak oils follow Beer's law there is little reason why stronger oils or concentrates should not.

Some of our results are put together in Table I, and shown graphically in Fig. 3. The normal concentration, 0·04 g. in 2·20 ml. = 18·18 g./l., is drawn in as a vertical line.

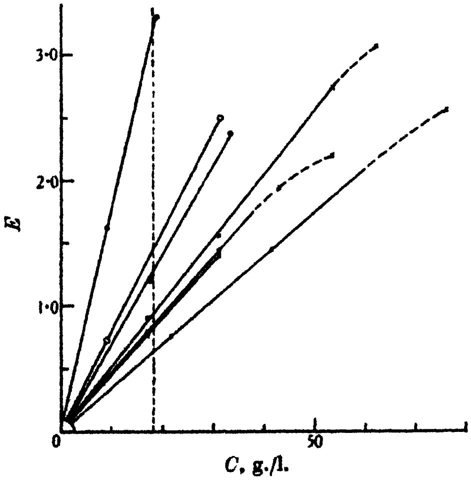

Fig. 3. *E* and concentration. o—o 618 mμ; •—• 603 mμ; ×—× 572 mμ.

It will be seen that the absorption maximum is a linear function of the concentration in all cases, falling off only when the volume of oil solution effects a considerable dilution of the reagent. There is nothing to indicate that the oil has any disturbing influence in the reaction, or rather, any disturbance is proportional to the amount of oil taken; the blue solution follows Beer's law.

The relation between tintometer readings and spectral absorption.

Blue values over the range of the tintometer and the corresponding maximum densities have been determined for a number of oils and concentrates (Table II).

Table I.

Oil no.	Maximum at *mμ*	Concentration g./l.	Layer cm.	ml. oil solution	$E_{\max}$
2*a*	572	21·8	1	0·1	0·76
		41·5	1	0·2	1·45
		76·3	0·5	0·4	2·56
4*c*	572	17·0	1	0·2	0·76
		31·2	1	0·4	1·41
4*a*	572	17·0	1	0·2	0·80
		31·2	1	0·4	1·44
		43·1	0·5	0·6	1·94
		53·5	0·5	0·8	2·20
4*b*	572	17·0	1	0·2	0·90
		31·2	1	0·4	1·56
		53·5	0·5	0·8	2·73
		62·4	0·5	1·0	3·06
2*b*	603	17·5	0·5	0·1	1·20
		33·4	0·5	0·2	2·38
7	603	8·90	1	0·1	1·62
		18·8	0·5	0·2	3·30
2*b*	618	8·94	1	0·1	0·73
conc.		31·4	0·5	0·4	2·52

Table II.

	Max. 527 *mμ*			Max. 603 *mμ*			Max. 618 *mμ*	
	E 572 *mμ*	B.V.		*E* 603 *mμ*	B.V.		*E* 618 *mμ*	B.V.
1*a*	0·44	3·5	1*b*	0·77	4·4	1	0·54	6·0
	0·84	5·5		1·47	8·3		0·55	6·5
	0·84	6·0	1*c*	0·77	4·7		1·04	10·0
	1·53	8·0		1·47	8·5		1·04	10·5
2	0·55	3·3	2	0·61	4·0		2·08	16·0
4	0·81	5·5		1·02	5·0	2	0·70	7·6
5	0·89	5·5		1·08	6·2		0·73	7·4
6	0·76	5·0		1·20	7·0		0·76	7·6
				1·21	7·1		1·07	9·6
				2·33	10·5		1·29	10·7
			3	1·06	5·5	3	2·20	12·0
				1·24	6·8		2·30	12·5
				1·28	7·3	4	0·37	4·3
				1·50	7·6		0·71	7·6
				1·51	7·4		1·36	12·0
				1·61	7·2	7	1·12	8·0
			4	0·85	4·8		2·15	12·0
				1·01	5·2		2·23	14·0
				1·28	6·1	8	0·59	5·0
				1·43	8·0		0·88	9·5
			5*a*	1·34	7·3		1·15	8·5
				1·48	7·9		1·15	10·0
			5*c*	1·80	9·5	10	1·15	8·6
				1·82	8·5		1·64	12·0
			7	1·60	7·2	11	0·86	6·5
				3·20	12·5	12	0·53	5·0
			8	0·84	8·0		0·80	9·0
			10	3·00	11·0		1·00	9·0
			13	0·37	2·4	14	0·35	3·3
				0·73	4·1		0·36	3·3
				0·72	4·3		0·51	4·6
				1·05	6·0		0·70	5·6
				1·43	7·3		0·72	5·8
				1·37	7·4		0·67	5·9
				1·46	7·6		0·96	7·4
				2·10	9·6		1·02	8·1
				2·86	11·3		1·40	9·7
				2·73	11·7		1·33	10·4
							2·04	13·4

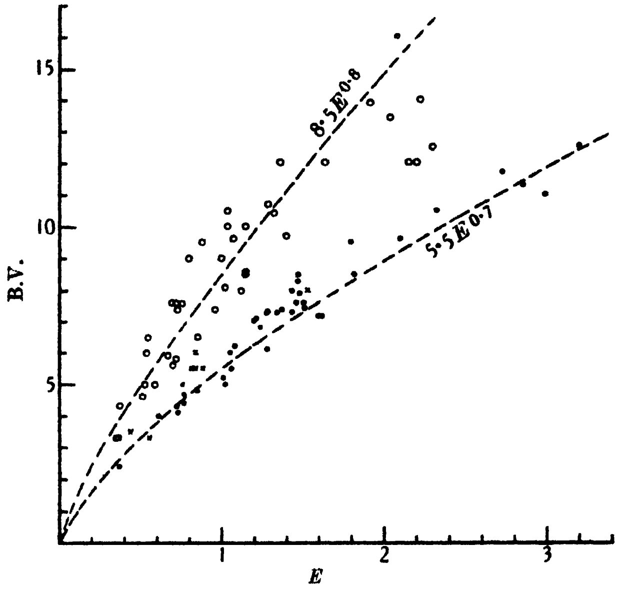

Fig. 4. *E* and B.V. o—o 618 *mμ*; •—• 603 *mμ*; ×—× 572 *mμ*.

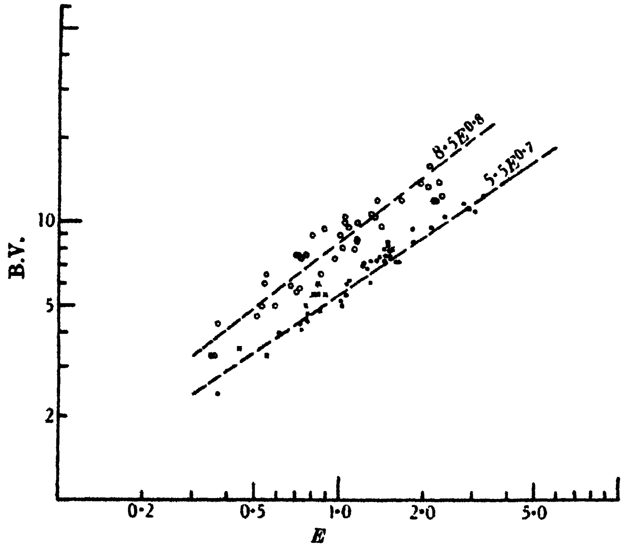

Fig. 5. *E* and B.V. on logarithmic scale.
o—o 618 *mμ*; •—• 603 *mμ*; ×—× 572 *mμ*.

As might be expected they do not show a linear relationship, either for oils or for concentrates; this appears clearly from Fig. 4. In Fig. 5 the values are plotted in a bi-logarithmic coordinate system. Here they will be seen to group around nearly parallel straight lines, one for each predominating absorption band. Straight lines in such coordinate systems are exponential functions. The line formed by the values for solutions with the maximum at 618 $m\mu$ has a slope corresponding to an exponent of about 0·8, those for 603 $m\mu$ of about 0·7. The lie of the lines give the two functions:

$$\text{B.V.} = 5{\cdot}5 . E^{0{\cdot}7} \text{ for oils, max. } 603\,m\mu.$$
$$\text{B.V.} = 8{\cdot}5 . E^{0{\cdot}8} \text{ for concentrates, max. } 618\,m\mu.$$

Solutions with maximum at 572 $m\mu$ give intermediate values, but the number of determinations does not allow any definite function to be derived. The functions above are to be regarded as average values, and do not claim great accuracy.

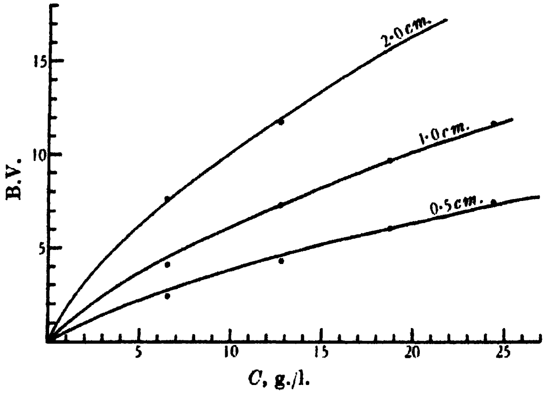

Fig. 6. B.V. and concentration. Cod liver oil.

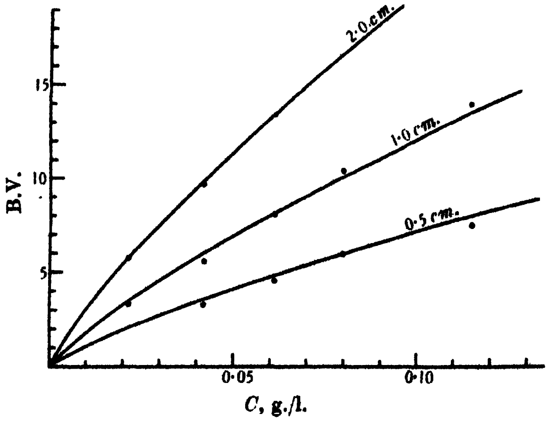

Fig. 7. B.V. and concentration. Halibut liver oil.

This is mainly because tintometer readings, even when carried out with the greatest care by experienced workers, are not very reliable. Variations in lighting compensation and also individual differences, or even differences in the same observer from day to day, all go to making the readings uncertain. It might be mentioned that the observers in question have had from three to five years' experience with the tintometer.

As the density is proportional to the concentration, the dilution curves for single oils or concentrates would naturally be expected to conform with exponential curves with the same exponents. In Figs. 6 and 7 values for various concentrations and thicknesses of layer for a medicinal cod liver oil and a strong halibut liver oil are drawn together with the corresponding exponential curves; the observed values will be seen to conform satisfactorily with these curves.

Blue value and thickness of layer.

As the blue solution follows Beer's law, it follows from the fact that the blue readings are not proportional to concentration and consequently not to density, that the blue reading cannot be proportional to the thickness of layer, but must follow a relation similar to that between blue reading and concentration or density. We have measured one ordinary and one potent oil in 0·5, 1 and 2 cm. cells; the measurements were made by three experienced observers. The resulting average readings are given in Table III. Graphically, in an ordinary coordinate system (Figs. 8 and 9) it appears clearly that the readings are not proportional. Curves for exponential functions with the exponent 0·8 for the potent oil, 0·7 for the normal oil, have been drawn in, the values will be seen to agree well with these curves. In a bi-logarithmic system they will be seen (Figs. 10 and 11) to lie approximately on straight lines, with slopes corresponding to the exponents mentioned.

Table III.

Cod liver oil			Halibut liver oil		
Layer cm.	Concentration	B.V.	Layer cm.	Concentration	B.V.
0·5	6·55	2·4	0·5	0·0418	3·3
	12·8	4·3		0·0613	4·6
	18·75	6·0		0·080	5·9
	24·4	7·4		0·115	7·4
1	6·55	4·1	1	0·0214	3·3
	12·8	7·3		0·0418	5·6
	18·75	9·6		0·613	8·1
	24·4	11·7		0·080	10·4
2·0	6·55	7·6		0·115	13·9
	12·8	11·3	2·0	0·0214	5·8
				0·0418	9·7
				0·0613	13·4

In Fig. 10 the lowest values do not agree well with the curve drawn which corresponds to the exponent 0·7. In fact it appears that very low values do not agree well with the exponential function, probably because the density becomes more influenced by the neutral caused by surface reflection, and less by the colour. This, however, is of little practical interest. Such low readings should be avoided anyhow as they are uncertain.

Why is the colour/concentration relation not linear?

The fact that the blue solution follows Beer's law, even in oil concentrations approaching three times those usually employed, and that the colour is not proportional to the layer of identical solutions, makes it obvious that the non-linear relation cannot be attributed to any "inhibitory" effect of the oil. The explanation must be purely physical, and must lie in the nature of the colours that are matched so as to appear alike to the eye.

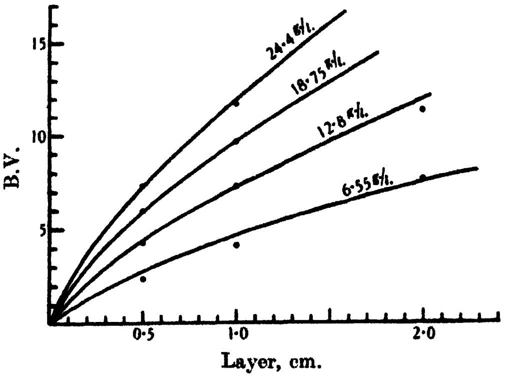

Fig. 8. B.V. and thickness. Cod liver oil.

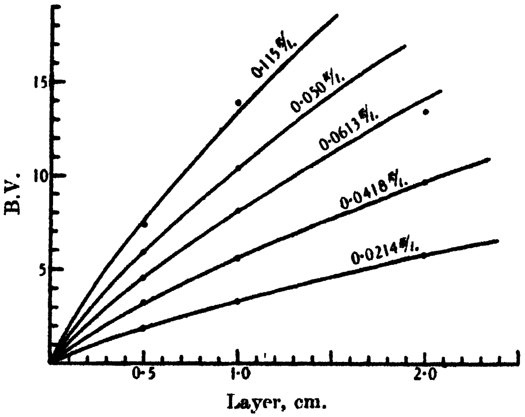

Fig. 9. B.V. and thickness. Halibut liver oil.

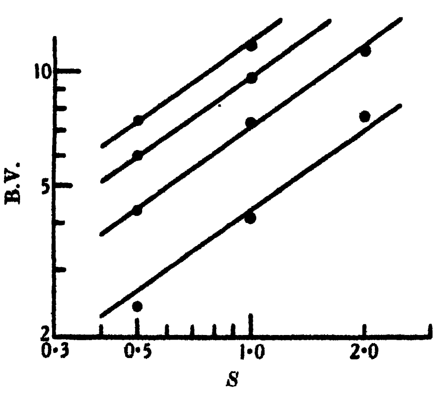

Fig. 10. B.V. and thickness on bi-logarithmic coordinates. Cod liver oil.

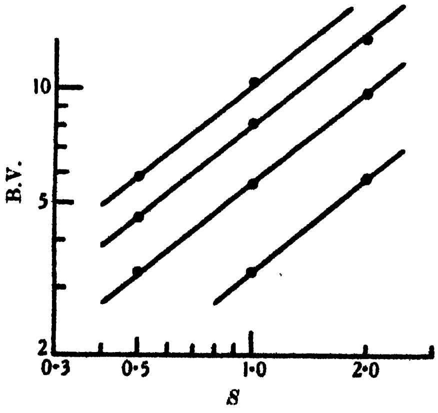

Fig. 11. B.V. and thickness on bi-logarithmic coordinates. Halibut liver oil.

For two colours to appear alike to the eye, it is necessary that two factors are more or less identical. First the colour quality must be the same. (Any given colour quality may be produced in innumerable ways by mixing three colours, provided that none of these can be produced by mixing the two others. Thus the tintometer can theoretically produce all known colour qualities.) If the colour quality is the same, to appear alike the two colours must transmit equal percentages of the incident light, reduced wave-length by wave-length to the sensitivity of the human eye. Such measurements of transmission may be made by computation, as has been done for the tintometer glasses by Gibson & Harris [1922], or more conveniently by means of a special photo-electric cell which has the same sensitivity curve as the eye. We have made photo-electric measurements of the transmission of the glasses of one of our tintometers, and for oil blue and concentrate blue solutions. The results should give straight lines in a linear/logarithmic coordinate system, and from Fig. 12 will be seen that they practically

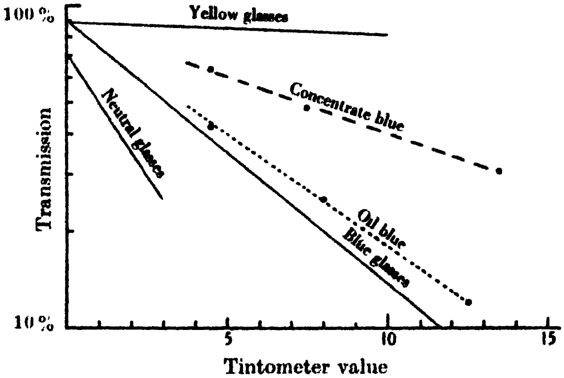

Fig. 12. Tintometer value and transmission.

do so. The points on the glass lines have been omitted as they lie practically on the line. It will be seen that the oil blue is only slightly more transparent than the glasses, bearing out the fact that ordinary oil blues need only yellow and little or no neutral for compensation. The yellow glasses practically only change the colour quality; they reduce the brightness very little.

Concentrate blues are much more transparent than the corresponding glass combination, so they need substantial reduction in brightness by neutral glasses.

The great physical difference of the colours is shown in a striking manner if the complete absorption curves of the 10 blue glass and oil and concentrate blue solutions which would need 10 blue to match are drawn together. In Fig. 13 the broken lines are the unreduced curves, the whole lines are the curves after they have been reduced to the sensitivity curve of the eye. The glass curve, measured on one of our tintometers, corresponds closely with that of Gibson & Harris [1922].

DISCUSSION.

As has been shown, the reason why the blue readings of antimony trichloride blue solutions are not proportional to concentration is purely physical (for a relation of a similar nature see Ferguson [1935]). It should therefore be possible to find the exact relation between density and blue reading through exact mathematical calculation, based on the laws of physics and physiological optics. This we shall not attempt here. For practical purposes the relations established earlier in this paper agree with experiment well within the limits of error of the methods, and the nature of the laws of light absorption makes it probable that the exact relation would have a similar form.

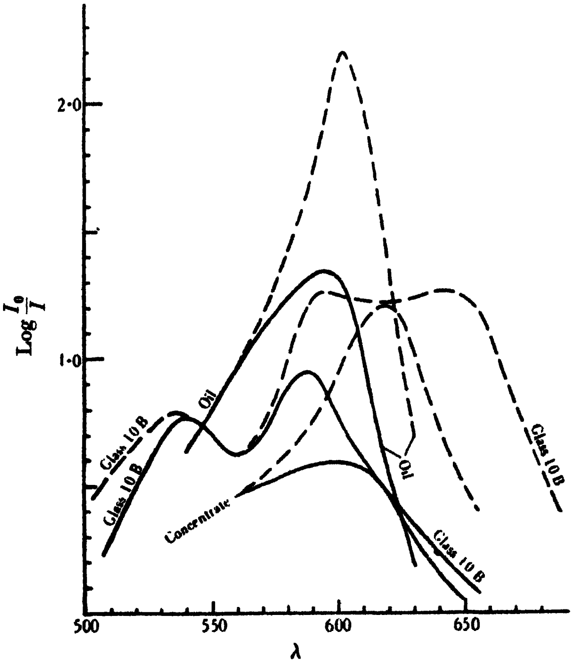

Fig. 13. Absorption curves for oil, concentrate and glass of tintometer value 10 blue.
- - - - Measured curves; —— Reduced curves.

The fact that the dilution curve follows an exponential function, together with the fact that variations in the amount of chromogen solution from 0·05 to 0·4 ml. do not affect the result means a considerable simplification of the determination of the blue value.

It is easy to understand why it has been necessary to measure the blue value at or near a given value, usually 6 blue. The "blue value" of a chromogenic substance must in reality be based on the concentration necessary to give an agreed reading (6) in the tintometer. This divided into the standard concentration, 18·18 g./l., and multiplied by the standard reading, 6, gives the blue value. Now, what has been said makes it possible to determine this concentration without diluting backwards and forwards to obtain a solution which gives 6 blue with 0·2 ml. + 2 ml. reagent. Any reading, at least between 4–5 and 10–12 blue, obtained with any amount between 0·05 and 0·4 ml. chromogen solution may be used for finding the "6-concentration".

If the concentration C gives a blue value B, the concentration c_6 which will give a blue reading 6 can be found by the equation

$$c_6 = C_B \cdot \left(\frac{B}{6}\right)^{\frac{1}{0\cdot 7}}$$

or 0·8 for concentrates.

This means that it will be possible to get readings on solutions from less than half as strong to six or eight times stronger than that which would give 6 blue with 0·2 ml.

The equation is, of course, easily worked out on a slide-rule, but if several are to be worked out a graph like Fig. 14 will be a great help. Here the reading b corresponds to a relative concentration k. A reading B will be found to correspond to a relative concentration C_B the value of which may be read from the graph, and the concentration which will give b is $\frac{1}{C}$ of the concentration which gave B. The graph is even easier to construct on bi-logarithmic paper (for instance Schleicher & Schull, Düren, paper no. 365½) where exponential curves, as has been said before, are straight lines.

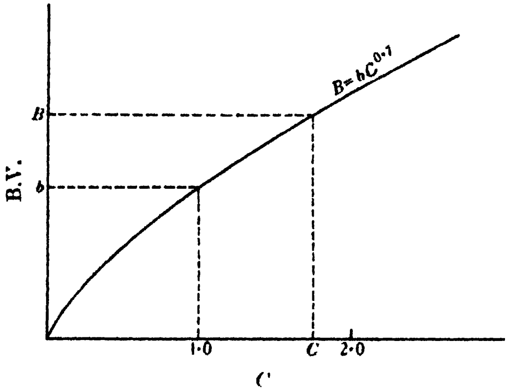

Fig. 14.

The relation between density and tintometer reading throws light on the relation between the blue value and the concentration of the substance, very probably vitamin A, which gives rise to the 618 and 603 $m\mu$ bands. If the extinction coefficient of the chromogen itself is unaltered when the band shifts, then it will be apparent from Figs. 4, 5 and 13 that a 603 $m\mu$ blue solution will contain nearly twice as much chromogen as a 618 $m\mu$ blue solution giving the same reading. Or, on the other hand, a solution with a given maximum density at 603 $m\mu$ will give a much lower reading in the tintometer than a solution with the same density at 618 $m\mu$.

Here again purely physical phenomena throw light on a question concerning the antimony trichloride reaction which has not so far found a satisfactory explanation, the relation between the blue value of oils and their concentrates. If the extinction coefficient, calculated for the oil, is unchanged by the preparation of the concentrate, then a blue value perhaps nearly twice that of the oil should be expected. Actually still higher values are found [v. e.g. Morgan & Pritchard, 1936]; this is because the extinction coefficient increases if no chromogen is lost, as the fatty matter inhibits the reaction. This side of the question will be dealt with in a paper which we hope will appear shortly.

SUMMARY.

Complete absorption spectra of the blue colours concerned in the antimony trichloride reaction have been taken.

The blue solutions, both of oils and concentrates, follow Beer's law, far beyond the concentrations encountered in practice. Only when amounts of oil solution above 0·5 ml. are used is a slight falling off observed, which is due to dilution of

the reagent and not to chemical inhibition by the oil. The amount of oil solution may safely be varied from 0·05 to 0·4 ml.

The well-known fact that the blue readings are not proportional to concentration is confirmed spectrographically. As the solutions follow Beer's law, the lack of proportionality is a purely physical phenomenon.

The following relations are found between E and blue reading:

$$\text{B.V.} = 5{\cdot}5 . E^{0{\cdot}7} \text{ for max. } 603\, m\mu.$$
$$\text{B.V.} = 8{\cdot}5 . E^{0{\cdot}8} \text{ for max. } 618\, m\mu.$$

Between a blue reading B obtained by a concentration C and a reading b obtained by a reading c there exists the relation

$$b = B . \left(\frac{c}{C}\right)^{0{\cdot}7} \text{ (0·8 for concentrates).}$$

The blue value, as read in the tintometer, of a solution with a given absorption maximum is 40–60 % higher when the band lies at 618 $m\mu$ than when it lies at 603 $m\mu$. Even if the reaction were not inhibited by the oil, a concentrate should therefore always give a blue value nearly twice that of the corresponding oil if they are measured at the same reading.

The authors wish to express their sincerest thanks to L. Aure and H. Hamre for much valuable help. Also to Dr O. Devik of the Christian Michelsen Institute, Bergen, for helpful advice, and to Prof. Helland-Hansen, of the Geophysical Institute, Bergen, for placing spectrograph and laboratories at their disposal.

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CCXLI. THE DETERMINATION OF VITAMIN C BY MEANS OF ITS INFLUENCE ON THE BODY WEIGHT OF GUINEA-PIGS.

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(*Received 14 July 1936.*)

MANY workers have found that young guinea-pigs lose weight and die when fed on a diet containing all substances known to be necessary for growth except vitamin C. Other young guinea-pigs fed on the same diet supplemented with vitamin C were found to live and increase in weight. This fact was made the basis for a comparison of the vitamin C content of different samples of apples by Bracewell *et al.* [1930]. Different groups of guinea-pigs were given graded doses of any one sample of apples for periods up to ninety days. The increase in weight and the intensity of the macroscopic lesions indicative of scurvy were used for the assessment of the vitamin C potency of the different doses. The daily doses of apple given to the different groups of animals were generally 3, 5, 10 and 20 g. respectively. The less potent samples gave evidence of their inferiority within four weeks of the beginning of the test. The more potent ones required often as much as eighty or ninety days to produce a difference in response to the different doses. Post mortem examination of the guinea-pigs, either after their natural death during the experimental period, or after they were killed at the end of the experimental period, revealed macroscopic lesions of an intensity which corresponded closely with their decrease or increase in body weight.

Harris & Ray [1932] compared graded doses of fresh ox suprarenal cortex with similarly graded doses of orange juice with respect to their influence both on the structure of the teeth and on the growth of guinea-pigs fed on a scorbutic diet. The respective doses of the two substances (0·25, 0·5, 1·0 and 2·0 g. suprarenal cortex and 0·75, 1·5, 3·0 and 6·0 ml. orange juice) gave practically equal average increases in weight of the groups of animals used and a very smooth curve of response was obtained relating increase in weight of the guinea-pigs to the dose of material given. The curve was very nearly logarithmic in shape for the negative increases in weight, but flattened somewhat for the positive increases. The ratio of the vitamin C potencies of the two substances obtained by this method confirmed the ratio obtained by the "tooth" method.

Thus it appeared that the increase in weight of young guinea-pigs was graded to the dose of vitamin C given, and it seemed to us that a method of estimation of vitamin C based on this property would have distinct advantages over the "tooth" method, provided that it could be carried out within a reasonable length of time, and provided also that it was found to give as great a degree of accuracy as the "tooth" method. It would have the obvious advantage of requiring no histological examination of the teeth with the attendant error involved in the assessment of structural changes, but, on the other hand, it would always be open to the well-known objections to which all tests based on increases in body weight are subject.

In our attempt to work out such a method we obtained a curve of response to graded doses of vitamin C (ascorbic acid, the International Standard of reference) but the curve, based on average responses of 12 animals in each group, was nearly smooth only after six weeks of feeding. Moreover, the degree of accuracy obtainable in the test, as calculated from the somewhat limited data available, was very similar to or slightly less than that obtainable by the "tooth" method as already worked out in this laboratory. Since our object was to find a simpler and possibly more accurate method than our present one, we did not carry on the experiment for the length of time, 80–90 days, recommended by Zilva. Hence we have no information as to the accuracy of the method as actually used by him. We have, however, concluded that the only advantage of a six weeks' growth test (increase in weight) lies in the avoidance of the histological examination of the teeth, whereas its disadvantages are (*a*) the greater length of time required, (*b*) the possibly lower accuracy and (*c*) the very great one of its not being specific for vitamin C.

If, on the other hand, greater weight is attached to the macroscopic symptoms of scurvy found in autopsies, then the assessment of results is as liable to subjective errors as the assessment in the "tooth" method. In our own experiment we drew up a scheme for assessing the macroscopic symptoms of scurvy found at autopsy and we obtained a curve of response relating the severity of scorbutic symptoms to dose of vitamin C given, which was very similar to the curve found for the increase in weight of the guinea-pigs.

METHOD.

The guinea-pigs were about 250 g. in weight at the beginning of the experiment. They were not bred in this laboratory, but were obtained from a reliable dealer. They were given large quantities of green food for three days before the experiment began. They were then given a scorbutic diet for the whole of the experiment. The diet consisted of:

Bran	45 %
Crushed oats	25 %
Dried skimmed milk	30 %

A fresh portion of this food was given each day. In addition each guinea-pig was given about 1 ml. of a good sample of cod liver oil twice a week directly into its mouth by pipette, to supply vitamins A and D. Fresh tap water was also given daily. The guinea-pigs were bedded on sawdust, three in each cage (dimensions about 12 in. × 18 in. × 8 in.). They were weighed twice a week.

Different doses of ascorbic acid were given to different groups of animals, each animal of any one group being given the same daily dose for six weeks. At the same time a certain fruit juice was tested also in order to see whether the response to different doses of fruit juice was graded to the dose in the same way (or to the same extent) as the response to different doses of ascorbic acid.

The doses given to the different groups were:

Group 1	No dose
2	0·125 mg. ascorbic acid
3	0·25 mg. ,,
4	0·5 mg. ,,
5	1·0 mg. ,,
6	2·0 mg. ,,
7	0·25 ml. fruit juice
8	0·5 ml. ,,
9	1·0 ml. ,,

The ascorbic acid was given as a solution in glass-distilled water. Fresh solutions were made each day and given to the animals immediately. They were of such concentrations that the required dose was contained in 0·5 or 1·0 ml. water. Each dose was given directly into the back of the guinea-pig's mouth by pipette. The doses were given to different guinea-pigs by four workers at once in order to minimize the time between making the solution and giving it to the guinea-pigs. Dosing was always finished within fifteen minutes of the making of the solutions. The two lower doses of fruit juice were diluted with an equal volume of water and given immediately. In the first part of the experiment only the two lower doses were tested. Each group of guinea-pigs consisted of six animals, but after about three weeks it was evident that the doses of fruit juice were rather low, so twenty-four more guinea-pigs were obtained. Four of these were added to each group receiving the three lowest doses of the Standard, six were added to the group receiving 0·5 ml. fruit juice and six constituted a new group and were given 1·0 ml. fruit juice. It was necessary to divide the fresh batch of guinea-pigs thus, in order to have controls on the Standard if there should prove to be a variation in the whole stock. Actually no such variation was detectable at the end of the experiment, and all the animals were therefore treated as one experiment.

RESULT.

It had been hoped that a period of four weeks' feeding would be sufficient to bring about a graded response to graded doses of vitamin C, i.e. to produce a reasonably smooth curve of response. When the first six animals in each group had been on experiment for four weeks, however, it was evident that the curve of response obtained by plotting the average increases in weight against the dose of ascorbic acid given was not at all a smooth one. It was therefore decided to make the experimental period six weeks, in the hope of obtaining a smoother curve, but not longer than this, for six weeks was considered the longest period that could be usefully employed in this test, since the method involving the histological examination of the teeth can be completed within four weeks.

The curve of response relating average increase in weight in six weeks to dose of vitamin C (Fig. 1).

The average weights of the groups of animals at weekly intervals during the experiment are given in Table I. The average increases in weight, collected in the last column, were calculated from those guinea-pigs only which survived the six weeks' experimental period. The increases in weight from the different doses of

Table I.

			Average weight of group of animals at end of week							Average increase in weight of those guinea-pigs (only) which survived six weeks
Group	No. of animals	Daily dose of supplement given	0 g.	1 g.	2 g.	3 g.	4 g.	5 g.	6 g.	
1	5	No dose	242·8	222·2	228·7 (2 died)	182·3	155·5 (2 died)	145·0 (1 died)	—	—
2	10	0·125 mg. ascorbic acid	245·3	239·2	254·3	248·0	219·1 (2 died)	150·7 (1 died)	143·4 (1 died)	−37·3
3	10	0·25 ,,	243·6	241·4	263·8	277·6	289·3	291·2	276·6	+33·0
4	10	0·5 ,,	242·1	239·9	256·9	268·0	289·6	306·2	304·9	+62·8
5	6	1·0 ,,	250·7	255·2	262·2	276·8	292·2	312·3	321·8	+71·2
6	6	2·0 ,,	239·8	239·0	263·5	267·5	291·0	308·5	324·0	+84·2
7	6	0·25 ml. fruit juice	244·2	239·6	247·5	261·3	220·5	175·5 (3 died)	163·3	−77·7
8	12	0·5 ,,	246·4	246·2	255·0	255·1	234·2	223·0	218·6	−25·5
9	6	1·0 ,,	244·8	245·2	258·5	268·7	274·5	271·5	288·0	+43·2

ascorbic acid were plotted against the doses given, and a curve drawn, by inspection, to connect the points (Fig. 1 A). It was apparently not logarithmic, but nevertheless a curve was drawn, plotting the mean increases in weight against the logs of the doses of ascorbic acid. This curve (Fig. 1 B) appeared to be more nearly logarithmic in shape, therefore a fresh curve was drawn relating the mean increase in weight to the log (log dose + 1) (unity was added to get rid of the minus sign of the log of the dose). This proved to be a straight line (Fig. 1 C) whose equation was $y = 74{\cdot}3 + 108{\cdot}2\,x$ where y = the mean increase in weight of the animals and x = log (log dose + 1). Hence the equation relating increase in weight to dose of ascorbic acid given is $y = 74{\cdot}3 + 108{\cdot}2$ log (log $10x$) where y = the increase in weight and x = dose of vitamin C in mg.

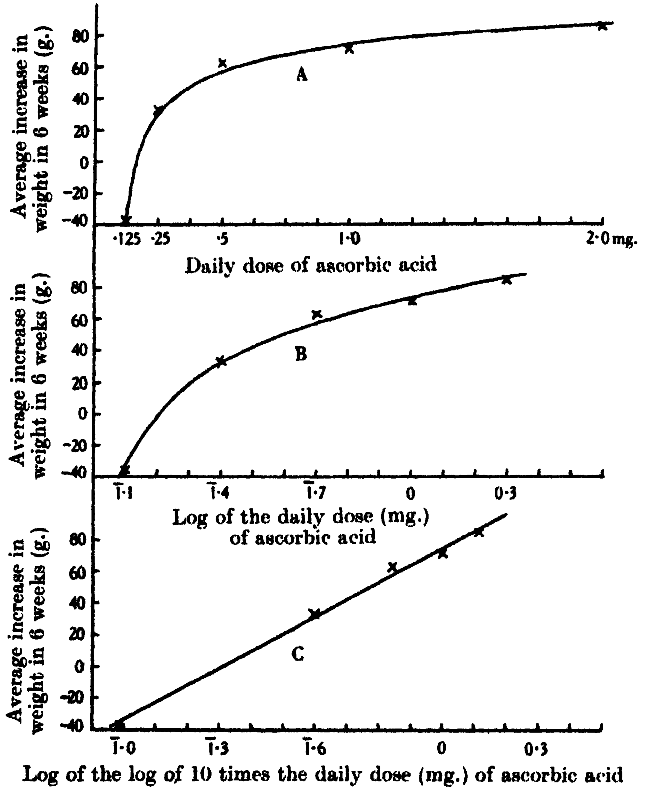

Fig. 1. The mean increases in weight of groups of guinea-pigs given graded doses of ascorbic acid daily for 6 weeks plotted against

(A) the dose of ascorbic acid (x) (equation $y = 74{\cdot}3 + 108{\cdot}2 \log(\log 10x)$);
(B) the log of the dose of ascorbic acid ($\log x$) (equation $y = 74{\cdot}3 + 108{\cdot}2 \log(x+1)$);
(C) the log of the log of 10 times the dose of ascorbic acid log (log $10x$) (equation $y = 74{\cdot}3 + 108{\cdot}2x$);

to show how the equation of the curve of response was obtained.

Confirmation of the curve of response.

The results obtained by the three doses of fruit juice confirm the shape of the curve of response obtained from the graded doses of ascorbic acid. Since the fruit juice was tested simultaneously with the doses of ascorbic acid which formed the curve of response, the results from the fruit juice may be compared directly with these results from the ascorbic acid.

Table II.

Daily dose of fruit juice (ml.)	Mean increase in wt. of guinea-pigs (g.)	Abscissa of curve corresponding to increase in wt.	Apparent potency of the fruit juice (mg. ascorbic acid per ml.)	No. of animals used in the test
0·25	-77·7	0·11	0·44	6
0·5	-25·5	0·13	0·26	12
1·0	+43·2	0·33	0·33	6

The result of $-77{\cdot}7$ g. increase in weight can only be used if the curve is extrapolated; it is subject to inaccuracy. If this result is omitted, then the weighted mean of the apparent potencies of the juice obtained from the two higher doses, $\frac{12 \times 0{\cdot}26 + 6 \times 0{\cdot}33}{12+6} = 0{\cdot}28$, may be taken as the vitamin C potency of the juice (mg. ascorbic acid per ml. juice). If, however, the result from the lowest dose is included, the result becomes 0·32 mg. ascorbic acid per ml. of juice and the potency of the juice may be stated to be, in whole numbers from either figure, 6 I.U. of vitamin C per ml.

Thus the curve of response may be used in future for comparing one dose of the Standard for vitamin C with one dose of the substance under examination in exactly the same way as a curve of response for vitamin A is used.

The accuracy obtainable by this method

The standard deviation of the increase in weight in six weeks of guinea-pigs given doses of vitamin C has been calculated from eight of the groups of animals used in this experiment. As the group given no dose died before the six weeks had ended, it could not be used for the calculation. The method we used was the one used by Coward [1932] in calculating the standard deviation of the results of vitamin A estimations. For the vitamin C tests, the standard deviation of a single determination σ was found to be 32·1. Therefore the probable error (1:1 chance) of the average increase in weight when ten animals are used on one dose is 6·76. The probable error of an estimation depends partly on the probable error of the average increase in weight and partly on the steepness of the curve of response relating increase in weight to dose of vitamin given. The probable error of the increase in weight in this test is high, which would make the probable error of an estimation high, but the curve of response is steep, which would make the probable error of the estimation low. Since the probable error of the mean response of a group of ten guinea-pigs is 6·76, the probable error of the difference between the responses of two groups of guinea-pigs is $6{\cdot}75 \times \sqrt{2} = 9{\cdot}56$. The probable error of an estimation is then determined by finding the abscissae of the curve of response corresponding to $y = \pm 9{\cdot}56$ about the value (mg. ascorbic acid) found by the experiment. For example, suppose a dose of fruit juice had been found to give a response of, say, 50 g. which was approximately equal to that given by a particular dose of the standard. The calculation of the probable error of the result, expressed as a percentage of the value found, is summarized in Table III A.

This is greater than the probable error of an estimation carried out by the "tooth" method when the result is obtained about the middle part of the curve of response.

Since, however, the curve of response in the growth test is not a simple logarithmic one the probable errors will be different for results obtained at different

Table III.

Mean increase in weight (g.)	Abscissa corresponding to the increase in weight	% values	Probable error of result %
	A.		
50	0·394	100	—
50 + 9·56 = 59·56	0·538	136	+36
50 − 9·56 = 40·44	0·306	77	−23
	B.		
0	0·161	100	—
0 + 9·56	0·179	111	+11
0 − 9·56	0·147	91	−9

parts of the curve. Suppose that the dose of fruit juice and the dose of Standard had each been sufficient just to maintain weight in the animals for the six weeks of the test, i.e. the mean increase in weight of the two groups of guinea-pigs had been approximately nil. The probable error of this result is given in Table III B.

The error of a test in which comparison is made with doses which just about produce maintenance in weight of the animals used is therefore relatively low.

Thus this test is more accurate when doses are chosen which just maintain weight in the guinea-pigs than when doses are chosen which bring about a large increase in weight. The dose which will bring about a mean increase in weight of 50 g. is only double the dose which will bring about a mean increase in weight of 10 g. (see Fig. 1 A). Thus there is a very narrow range of effective doses for a vitamin C test. One could not expect often to find that one had chosen the dose that merely produced maintenance of weight, i.e. the one that gave the most accurate result. Hence it is perhaps fairest to state the accuracy of a result obtained by this method as a probable error of +11 to +36 or −9 to −23 %, or an average of +23 and −16 % which is very similar to the error at the middle part of the curve in the "tooth" method.

Thus, it is concluded that the increase in weight of guinea-pigs given abundance of all other substances known to be necessary for growth is dependent on the amount of vitamin C given. This fact may be made the basis for a method of estimation of vitamin C with the procedure, so far found necessary for all biological estimations, of carrying out a simultaneous test of the standard in every estimation made.

Assessment of macroscopic lesions of scurvy.

At autopsy scorbutic symptoms were looked for in the elbow and knee joints (haemorrhages) and in the ribs (swollen costochondral junctions). Different degrees of severity were denoted by the numbers 1–4. The state of each part was assessed separately and the condition of each guinea-pig assessed as the average of the three figures given to the various parts. The average degree of scurvy developed in each group was then calculated. All the animals in group 1 (given no supplement) died within the six weeks of the test and most of them had developed only slight signs of scurvy. The other groups developed scurvy to an extent proportional to their deprivation of vitamin C in a curvilinear relationship. The results are collected in Table IV.

A calculation of the relationship between the average intensity of scurvy developed in six weeks and the daily dose of vitamin C given was made in the same way as the calculation relating average increase in weight and dose of vitamin C given (Fig. 2 A, B, C). The relationship was found to be expressed by

Table IV.

Group	Number of animals	Daily dose of supplement given	Average degree of scurvy developed
1	5	No dose	1·0
2	10	0·125 mg. ascorbic acid	1·83
3	10	0·25 ,,	1·55
4	10	0·5 ,,	0·67
5	6	1·0 ,,	0·33
6	6	2·0 ,,	0·25
7	6	0·25 ml. fruit juice	2·54
8	12	0·5 ,,	1·59
9	6	1·0 ,,	0·75

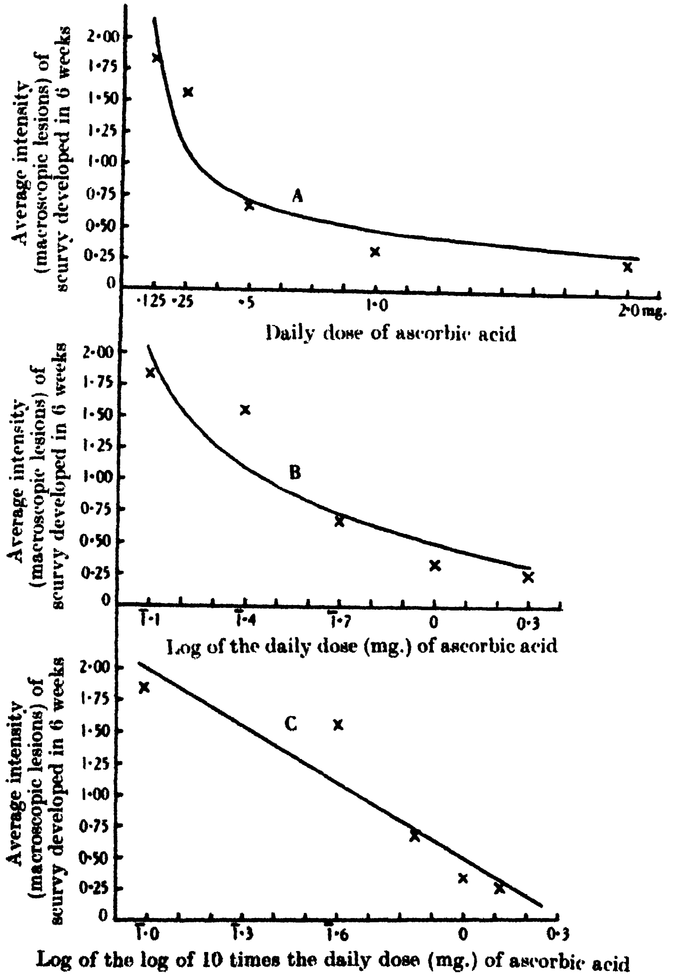

Fig. 2. The average intensity of scurvy developed in groups of guinea-pigs given graded doses of ascorbic acid daily for 6 weeks plotted against

(A) the dose of ascorbic acid (x) (equation $y = 0·49 - 1·50 \log (\log 10x)$);
(B) the log of the dose of ascorbic acid log (x) (equation $y = 0·49 - 1·50 \log (x+1)$);
(C) the log of the log of 10 times the dose of ascorbic acid log (log $10x$) (equation $y = 0·49 - 1·50x$);

to show how the equation of the curve of response was obtained.

the equation $y=0{\cdot}49-1{\cdot}50 \log (\log 10x)$, where y = the intensity of scurvy developed (as measured by the particular scheme adopted) and x = the daily dose (in mg.) of ascorbic acid given. How nearly the points fit this curve may be seen in Fig. 2 A. It is evident that four of the five points fit a curve slightly below this one remarkably well but the fifth point raises the whole curve.

By substituting in the equation $y=0{\cdot}49-1{\cdot}50 \log (\log 10x)$ the values of y obtained by giving the three different doses of fruit juice, the following values were found:

0·25 ml. fruit juice contained 0·110 mg. ascorbic acid.
0·5 ml. fruit juice contained 0·164 mg. ascorbic acid.
1·0 ml. fruit juice contained 0·469 mg. ascorbic acid.

These three results give the potency of the juice as 0·44, 0·33 and 0·47 mg. respectively of ascorbic acid per ml. The average, weighted according to the number of animals in each group, is 0·39 mg. ascorbic acid per ml. of juice. Therefore the juice contains about 8 I.U. of vitamin C per ml.

A simpler calculation for the potency may be made in the following way.

(*a*) 1 ml. fruit juice gave slightly less protection than 0·5 mg. ascorbic acid.

Therefore 1 ml. fruit juice appears to contain about 8 I.U. of vitamin C.

(*b*) 0·5 ml. fruit juice gave the same amount of protection as 0·25 mg. ascorbic acid.

Therefore 1·0 ml. fruit juice appears to contain 10 I.U. of vitamin C.

(*c*) 0·25 ml. fruit juice gave less protection than 0·125 mg. ascorbic acid.

Therefore 1·0 ml. fruit juice appears to contain about 7 I.U. of vitamin C.

The average of the three results (*a*), (*b*) and (*c*) is about 8 I.U. of vitamin C per ml. of juice, which is the same result as that obtained by the more elaborate calculation and may be considered a fair confirmation of the value, 6 I.U. of vitamin C per ml. of juice, found by the method based on increase in weight. Therefore, the estimation of the vitamin C potency of a substance obtained by comparing the average intensity of scurvy developed in the animals given doses of that substance with that in animals given doses of the International Standard at the same time, gives the same result as a comparison of the increases in weight of the same animals during the experiment.

The amount of ascorbic acid present in the fruit juice was not determined by the chemical method as sulphur dioxide had been used for its preservation.

Comparison of this method with the "tooth" method.

The method of estimating vitamin C by its influence on the body weight of guinea-pigs during a period of six weeks is no more accurate than the method of estimating it by its influence on the histological structure of the teeth during a period of two weeks. It has two advantages over the "tooth" method, (*a*) it is independent of the subjective errors of assessment of the amount of scurvy developed, (*b*) it does not involve the rather skilled technique of cutting the sections of the teeth. On the other hand, the "tooth" method of estimation can be completed within a period of four weeks and also it has the very great recommendation of being based on a reaction specific for vitamin C. It seems to us, therefore, that the "tooth" method is still very much to be preferred to the "increase in weight" method as worked out by us.

SUMMARY.

A method for the estimation of vitamin C has been worked out on the basis of its influence on the body weight of guinea-pigs. The method is very similar to the method of estimating vitamin A which was worked out in this laboratory.

Different groups of guinea-pigs were given daily doses of 0·125, 0·25, 0·5, 1·0 and 2·0 mg. of ascorbic acid (International Standard), each guinea-pig in any one group being given the same daily dose. A diet containing abundance of all other substances known to be necessary for growth was given *ad lib.*, a fresh portion each day. The guinea-pigs were weighed twice a week. When the test had been carried on for six weeks a fairly smooth curve of response was obtained relating increase in weight to dose of vitamin C given. In any period shorter than six weeks, the curve of response was much less smooth. The curve of response was represented by the equation $y = 74{\cdot}3 + 108{\cdot}2 \log (\log 10x)$.

The severity of the scurvy developed by the animals during the test also bore a curvilinear relationship to the dose of vitamin C given. A fairly good curve of response, $y = 0{\cdot}49 - 1{\cdot}50 \log (\log 10x)$, was obtained.

A sample of fruit juice examined by a test carried out simultaneously with the tests on the ascorbic acid (International Standard) was found to contain 6 I.U. of vitamin C per ml. as estimated by the increase in weight of the animals, and 8 I.U. per ml. as estimated by the severity of scurvy developed. This was considered to be fairly good agreement.

The accuracy of the "increase in weight" method has been calculated. It is not greater than the accuracy of the "tooth" method.

The relative merits of the two methods have been discussed. It is concluded that the "tooth" method has distinct advantages over the "increase in weight" method as carried out in the experiment described in this paper.

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CCXLII. VARIATIONS IN THE CAROTENE AND VITAMIN A VALUES OF THE MILK FAT (BUTTER) OF CATTLE OF TYPICAL ENGLISH BREEDS.

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EARLIER experiments have shown that the colour and vitamin A content of butter can be maintained at the high levels usually regarded as peculiarly characteristic of summer even in the winter period of stall feeding by the addition of artificially dried grass to the ration [Gillam *et al.*, 1933; Watson *et al.*, 1933]. Similar results have also been obtained with A.I.V. silage [Watson *et al.*, 1934]. As an extension of these experiments it was decided to obtain data on the variation of the carotene and vitamin A contents with season, breed and stage of lactation of the milk fat of cows kept under typical English conditions of feeding and management. Studies of some of these variations in the milk of English cows have previously been made using either colorimetric or biological methods of assay [Dann, 1933; Booth *et al.*, 1933: 1934], but at the time this investigation was commenced, no comprehensive study of the variations in the carotene and vitamin A contents of milk, using the more precise spectrophotometric methods of assay, had been made. Since then, however, thorough investigations of the fat of milk produced under American conditions of management have been carried out [Baumann *et al.*, 1934], using the spectrophotometric method developed earlier [Baumann & Steenbock, 1933].

Details of experiment.

For the experiment, two cows were selected from each of the main dairy breeds—Shorthorn, Ayrshire, Friesian and Guernsey. All the cows calved during October or November 1934, and seven of them were part of a large herd, receiving therefore uniform management and feeding. The remaining cow, an Ayrshire—Heather Lass—belonged to a neighbouring herd in which "outwintering" of cattle is the usual practice.

The particulars concerning the cows were as follows:

Breed	Name	No. of calves previous to experiment	Calving date
Shorthorn	Annette	3	31. x. 34
	Cherry	1	13. x. 34
Ayrshire	Ninna	7	22. xi. 34
	Heather Lass	4	28. xi. 34
Friesian	Dairymaid	4	20. x. 34
	Pearl	3	3. x. 34
Guernsey	Eva	2	6. x. 34
	Nellie	2	28. x. 34

During October, November and December, the cows received a winter ration of hay, kale and concentrates, the kale being fed at the rate of 30–40 lb. per day. From January 1935 to the end of April the kale was replaced by mangolds. The cows were turned out in April, but owing to a wet cold spell, there was very little available grass. During May and June, the cows had access to a plentiful supply of good quality pasture. In July, as a result of a drought, the quality and quantity of the grass fell off rapidly, and after the second week the fields were bare and burnt, and supplementary feeding with hay and concentrates was necessary.

Collection of butter samples. Immediately following calving, the colostrum was collected, as completely as was possible practically, during the first 2–3 days. With some difficulty, butter was prepared from the material after it had settled for a few days. At monthly intervals thereafter, the total milk from an afternoon and following morning's milking was collected, separated, allowed to "ripen" and then churned. The butter was then immediately clarified and the butter fat stored in a refrigerator until the necessary determinations could be carried out. In the later stages of lactation, it was necessary to collect two days' supply of milk in order to obtain sufficient for the making of the butter.

Determination of carotene and vitamin A in the butters. Samples (20–25 g.) of the dry filtered butter fats were saponified and the carotene and vitamin A determined in the unsaponifiable matter by absorption spectra methods, following the technique previously described [Gillam, 1934]. The gross absorption of the unsaponifiable matter in chloroform at 455–460 mμ, less a constant 6 % correction for xanthophyll, was converted into absolute carotene values by comparison with the standard absorption of pure β-carotene, $E^{1\%}_{1\,\mathrm{cm}}$ 463 mμ (chloroform) = 2200 [Gillam, 1935]. The absorption at 328 mμ (Hilger E_3 quartz spectrograph), after correction for that due to carotenoids, was similarly converted into percentages of vitamin A by making use of the value of $E^{1\%}_{1\,\mathrm{cm}}$ 328 mμ = 1600 for the purest vitamin A distillates [Carr & Jewell, 1933]. The values so obtained are relative and, provided that vitamin A is not concentrated further, are absolute also. (Karrer & Morf [1933] have reported a value of $E^{1\%}_{1\,\mathrm{cm}} = 1700$.)

Discussion of results.

The results of the examination of the butters of each cow throughout the whole period of lactation are shown graphically in Figs. 1 and 2. In considering the data, it must be emphasized that only two cows of each breed were used, and these exhibit marked individual variations, particularly in regard to carotene content, the vitamin A values showing closer agreement. As a result, the variations in values in the different breeds are only significant when the differences are very marked. This is shown clearly in contrasting the Shorthorn and Ayrshire groups. In a previous experiment [Watson *et al.*, 1934] the relative positions of these two breeds were the inverse of those of the present investigation. These variations, however, do not affect the general trend of the results. It will be seen that the butters show the normal decrease in their vitamin A and carotene values as the winter proceeds, reaching a minimum about the end of March [Gillam *et al.*, 1933]. With the advent of spring and fresh grass, the values rise sharply and continue until they reach the high values characteristic of summer butters. An interesting example of this well-known dependence of the quality of the butter on the supply of carotene-rich food is shown in the butters from the Ayrshire cow, Heather Lass. Whilst all the other cows in the experiment were stall-fed on similar rations, this cow was "outwintered", i.e. allowed access to pasture throughout the winter. The results are shown in the higher carotene values of the resulting butter and more particularly in the vitamin A values, which

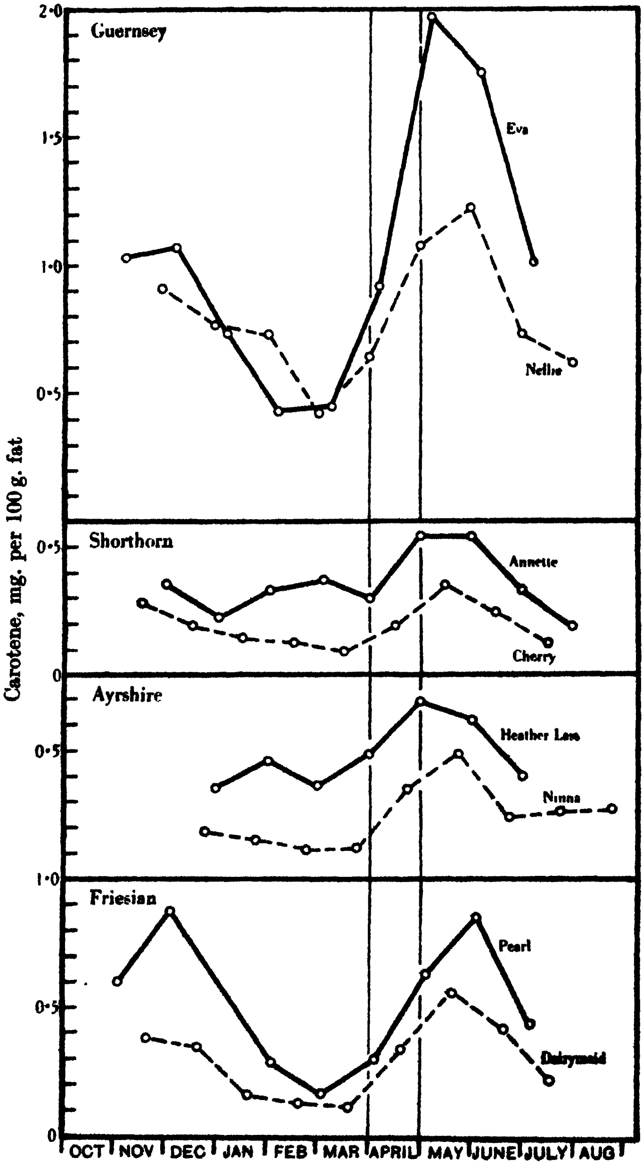

Fig. 1. Variation in the carotene content of butter fat during lactation of the cow. Oct. to March, stall-feeding. April, grazing but grass scarce. May and June, grass good and plentiful. July and Aug., drought; grass scarce and poor.

(All cows calved one month prior to date of first sample.)

are consistently much higher than those of the other Ayrshire cow while this was stall-fed (Fig. 2).

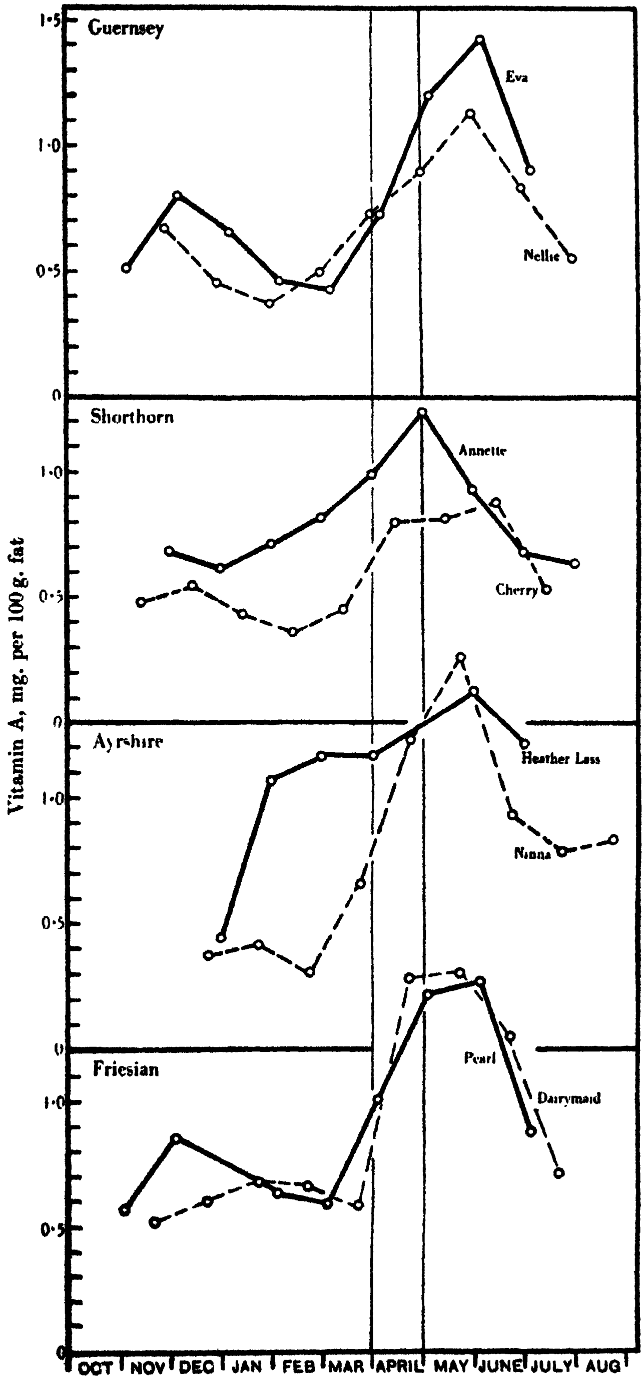

Fig. 2. The variation of the vitamin A content of butter fat during lactation of the cow. Oct. to March, stall-feeding. April, grazing but grass scarce. May and June, grass good and plentiful. July and Aug., drought; grass scarce and poor.

(All cows calved one month prior to date of first sample.)

Average values for typical English milks.

The carotene and vitamin A values of the milk fats from the individual cows have been collected and summarized in Table I together with the average values for each breed.

Table I. *Summarized values for all four breeds.*

Breed		Gross average value	Winter average	Summer average	Minimum values	Maximum values	Vitamin A/carotene ratio
Shorthorn	Carotene	0·27	0·25	0·29	0·09	0·54	2·5 : 1
	Vitamin A	0·68	0·58	0·84	0·36	1·24	
Ayrshire	Carotene	0·36	0·27	0·41	0·11	0·69	2·4 : 1
	Vitamin A	0·85	0·66	1·18	0·30	1·42	
Friesian	Carotene	0·40	0·35	0·47	0·11	0·85	2·25 : 1
	Vitamin A	0·90	0·61	1·21	0·52	1·50	
Guernsey	Carotene	0·92	0·73	1·14	0·42	1·97	0·80 : 1
	Vitamin A	0·75	0·54	0·95	0·37	1·42	

NOTE 1. All values are expressed in mg./100 g. fat. (For bases of calculation, cf. p. 1729.)

NOTE 2. The samples were divided into winter and summer samples arbitrarily, October–March being called "winter" and April–September being called "summer".

The Ayrshire values are a little higher than would otherwise be the case owing to the fact that one cow—Heather Lass—had access to fresh winter grass.

The Shorthorn values are lower than were expected from previous work. This is shown by the figures given later in this paper (Table IV) for larger groups of Shorthorn cows taken at different stages of lactation. The average values here are 0·50 mg./100 g. of carotene and 1·01 mg./100 g. of vitamin A. These values are not directly comparable with the values in Table I which include a large number of winter samples. On account of the fact that the gross growth-promoting activity of the butter fats is due to two different entities (vitamin A and carotene), the relative amounts of which vary both from breed to breed and, to a less extent, from cow to cow, a better appreciation of the relative values of the fats is obtained by expressing the total vitamin A activity in terms of biological units calculated from the known amounts of carotene and vitamin A present. The results are expressed arbitrarily in β-carotene units (γ per g.) on the basis of equal potencies for pure vitamin A and pure β-carotene [Moore, 1933]. The resulting values have the advantage of presenting a relative set of figures on a uniform basis for the four breeds; it is not claimed, however, that the same values would be found by actual biological test.

Table II. *Calculated growth-promoting activities of the butter fats in* γ *per g.* (*β-carotene units*).

Breed	Gross average	Summer average	Winter average	Maximum	Minimum
Shorthorn	9·5	11·3	8·3	17·8	4·7
Ayrshire	12·1	15·9	9·3	20·8	4·1
Friesian	13·0	16·8	9·6	23·2	6·9
Guernsey	16·7	20·9	12·7	31·7	8·8

These figures make the Guernsey appear the best of the four breeds as far as the gross content of vitamin A-active substances in the milk fat is concerned, a result which is not consistent with the finding that Shorthorn and Guernsey butters have approximately identical biological activities throughout the year [Booth *et al.*, 1934]. In this connection, it has recently been shown that pure carotene fed in vitamin A-free butter fat is apparently 2–3 times less effective than when fed in cottonseed oil [Kraybill & Shrewsbury, 1936], a result which may explain the observation that the vitamin A of butter is several times more effective biologically than the carotene [Booth *et al.*, 1934]. Thus it would seem that carotene is not utilized at all efficiently from butter fat by the rat, and this would tend to lower the apparent gross vitamin A activity (as determined biologically) of Guernsey butters, which owe more of their activity to carotene than to vitamin A. Thus, though Guernsey butters commonly contain more of the vitamin A-active substances than butters of other breeds, the question whether the human organism, unlike that of the rat, can absorb the carotene as effectively as the vitamin A must still remain open.

Colostrum.

The carotene and vitamin A values of the colostral milk (bulked samples collected during the first two days after calving) are markedly greater than those of normal milks, thus serving the purpose of providing the calf with a highly concentrated food in the first few days of life. The data on colostrum compared with milks from the same cows a month after calving are shown in Table III. The colostrum values, having been obtained on samples collected over two days, are not as high as those obtained on the first day's colostrum only.

Table III. *Carotene and vitamin A of colostrum.*

Date	Cow	Breed	Carotene mg./100 g. fat	Vitamin A mg./100 g. fat
Oct. 20	Dairymaid	Friesian	3·40	1·24
Nov. 20	,,	,,	0·38	0·52
Nov. 22	Ninna	Ayrshire	4·60	2·96
Dec. 22	,,	,,	0·18	0·37
Nov. 30	Heather Lass	,,	4·21	3·13
Dec. 30	,,	,,	0·35	0·44
Oct. 28	Nellie	Guernsey	3·61	1·13
Nov. 28	,,	,,	0·91	0·67
Oct. 13	Cherry	Shorthorn	2·90	3·51
Nov. 13	,,	,,	0·28	0·48
Oct. 31	Annette	,,	3·80	1·37
Nov. 30	,,	,,	0·35	0·68

The high vitamin A value of cow's colostrum has previously been demonstrated biologically by Dann [1933] in England, and spectroscopically by Semb *et al.* [1934] in America. The latter authors have shown that the high potency of the colostral milk persists for only a few days after the calf is born before dropping to a more normal value, a fact which is fully confirmed by the present results.

End of lactation effect.

The graphs (Figs. 1 and 2) show the variation in carotene and vitamin A content of the fat of each of the cows in the experiment throughout the whole of lactation. The marked decrease in these values towards the end of lactation, which coincided with the known scarcity of grass occasioned by the drought of July–August 1934, made it necessary to carry out experiments to decide whether the drop in quality of the milk was in fact due to a low-carotene diet, or was a genuine end of lactation effect. In order to decide between these two alternatives, bulked fat from 5 groups of 6 cows, all on the same diet, and at the same season but at different stages of lactation, was examined in the early winter of 1935. The following results were obtained, and show clearly that there is no obvious drop in the carotene and vitamin A of milk fat as the cow nears the end of lactation. This is in agreement with the results obtained by Treichler *et al.* [1935].

Table IV. *Carotene and vitamin A values of milk fat of Shorthorn cows at different stages of lactation.*

Sample	Weeks after calving	Date	Carotene mg./100 g. fat	Vitamin A mg./100 g. fat
A	10	27. xi. 35	0·46	1·10
D	15	28. xi. 35	0·45	0·95
B	19–23	29. xi. 35	0·58	1·06
E	29	20. xi. 35	0·54	0·93
C	30	29. xi. 35	0·46	1·03
		Averages	0·50	1·01

SUMMARY.

The carotene and vitamin A values of the milk fats (butter) of each of eight cows have been determined spectrophotometrically at monthly intervals from the day of calving to the end of lactation. Four groups of two cows were used, each group representing a typical English breed (Shorthorn, Ayrshire, Friesian and Guernsey), and all were treated alike, being stall-fed during the winter and pasture-fed in spring and summer under typical English conditions of management. The results show that individual variations among cows of the same breed are large but that, apart from the abnormally high values of colostrum, the carotene and vitamin A values of the butters are much more dependent on diet than on stage of lactation. Maximum, minimum and mean values for each breed are recorded.

Comparison of the summations of carotene plus vitamin A values places the breeds, in order of vitamin A activity of the butters, Guernsey > Friesian > Ayrshire > Shorthorn. The results of this and previous work indicate that the differences between butters of the last three breeds are scarcely significant.

The thanks of the authors are due to the Research Council of Messrs Imperial Chemical Industries Ltd. for a grant and to Mr G. V. Cawthorne for assistance in the analysis of the butters.

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CCXLIII. THE ISOMERIZATION OF CAROTENES BY CHROMATOGRAPHIC ADSORPTION.

I. *PSEUDO*-α-CAROTENE.

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(*Received 21 July 1936.*)

In an earlier examination of the carotene of butter by the chromatographic adsorption method [Gillam & Heilbron, 1935] it was found that the pigment separated readily into two clearly-defined coloured zones when adsorbed on alumina from light petroleum solution. The colour, location and general appearance of the zones together with the absorption spectra of the separately eluted pigments indicated that, in addition to β-carotene, appreciable amounts of the α-isomeride were also present. α-Carotene has since been independently detected in butter, using magnesium oxide as adsorbent instead of alumina [Strain, 1935]. A comparison of the adsorption of butter carotene on each of these two adsorbents has since shown that chromatographic analysis on alumina makes the α-carotene content of butter appear much greater than when magnesium oxide is used.

In order to explain these anomalous results we have carried out adsorption experiments on pure β-carotene using, primarily, aluminium oxide as adsorbent. The results show that the process of adsorption itself separates β-carotene into two zones of pigment having properties exactly comparable with those obtained when gross butter carotene is used. Subsequent experiments have shown that the lower zone contains a new carotenoid pigment having absorption maxima (in the visible) different from those of β-carotene but identical with those of α-carotene. It is thus clear that when the carotene fraction of butter is adsorbed on alumina the second pigment is not wholly α-carotene, as was at first thought, but contains a considerable amount of a new compound.

These phenomena have already been reported briefly [Gillam & El Ridi, 1935, 2], but since then, starting with pure β-carotene, we have prepared the new pigment, which we propose to call *pseudo*-α-carotene. The present paper describes its preparation and properties.

Table I summarizes these properties in comparison with those of α- and β-carotene examined under the same conditions [cf. also, Kuhn & Brockmann, 1931; Kuhn & Lederer, 1931, 1, 2; Karrer & Walker, 1933; Strain, 1934, 2].

The probable structure of pseudo-*α-carotene.*

The method of preparation of *pseudo*-α-carotene from β-carotene and the analytical data indicate that the substance is a hydrocarbon carotenoid, of formula $C_{40}H_{56}$, produced by isomerization of β-carotene.

Preliminary feeding tests on rats, for which we are indebted to Mr A. L. Bacharach of Glaxo Laboratories, Ltd., show clearly that the substance possesses strong vitamin A activity when fed at the rate of 2γ per rat per day. From this it can be inferred that the compound contains at least one structural unit made up of a β-ionone ring and four ethylene linkages conjugated with it, this unit being—as far as our knowledge goes—specifically associated with vitamin A

Table I. *Properties of* pseudo-α-*carotene.*

Compared with those of the α- and β-carotenes.

	pseudo-α-Carotene	β-Carotene	α-Carotene
Formula	$C_{40}H_{56}$	$C_{40}H_{56}$	$C_{40}H_{56}$
Melting-point	166° (uncorr.)	182° (uncorr.)	183·5° (uncorr.)
Rotation	Nil	Nil	+377° (benzene)
Absorption maxima ($m\mu$):			
Carbon disulphide	507, 477	514, 484	507, 476
Chloroform	486, 456	491, 463	487, 456
Petroleum (70–80° B.P.)	477, 446	484, 452	477, 447
Pyridine	493, 461	—	—
Ethyl alcohol	478, 447	—	—
Antimony trichloride:			
Colour	Blue	Blue	Blue
Absorption maxima ($m\mu$)	591 only	591, 541	591, 541, 492
Analyses. Found:*			
Carbon %	89·3	89·5	89·0
Hydrogen %	10·0	10·1	10·6
Partition, 90% methyl alcohol-petrol	Epiphasic	Epiphasic	Epiphasic
Hydrogenation (double bonds)	10·9	10·9	11·0
Adsorption on alumina	Slowly produces another zone above the main *pseudo*-α-carotene zone (β-carotene?)	Slowly forms a lower zone of *pseudo*-α-carotene	Slowly produces a lower zone below the main zone, different from *pseudo*-α-carotene
Adsorption affinity	Less than that of β-carotene	Greater than either α- or *pseudo*-α-carotene	Less than β- and similar to that of *pseudo*-α-carotene

* $C_{40}H_{56}$ requires 89·55, 10·45.

activity [cf. Kuhn & Brockmann, 1935]. Quantitative hydrogenation of the pigment shows that it contains a total of eleven ethylene linkages but absorption spectra data indicate that only ten of these are conjugated. The absorption maxima in the visible are identical with those of α-carotene, lutein and β-oxycarotene, and the chromophore responsible for the absorption should therefore be the same in each case, i.e. one double bond in a β-ionone ring and nine others in a straight chain conjugated with it [Karrer, Morf & Walker, 1933; Kuhn & Brockmann, 1932, 1; Karrer, Zubrys & Morf, 1933; Kuhn & Lederer, 1931, 1]. The eleventh double bond must be isolated from the conjugated system (cf. α-carotene Formula I).

Owing to the similarity of the compound to α-carotene the question arises whether, in fact, it is identical or not. The evidence is as follows:

(*a*) *pseudo*-α-Carotene melts at 166° (uncorr.) ± 1° only in four different crystalline preparations. Contrast α-carotene, M.P. 187° (corr.) [Karrer & Walker, 1933], and our own specimen (*vide supra*), M.P. 183·5° (uncorr.).

(*b*) It has no detectable rotation; contrast α-carotene $[\alpha]_{Cd} = +380°$. This absence of optical activity, however, might be due to racemization which is to be expected from the manner of its preparation from inactive β-carotene.

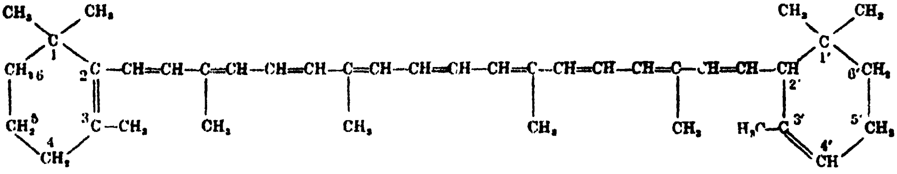
(*c*) By adsorption on alumina *pseudo*-α-carotene is partially re-converted into a substance identical with, or closely similar to, β-carotene.

Genuine α-carotene, on the other hand, behaves quite differently on adsorption, for whereas *pseudo*-α-carotene is converted into a pigment having absorption maxima at longer wave-lengths, α-carotene is converted into another pigment with absorption maxima at shorter wave-lengths.

Pigment	Adsorption on alumina	Absorption maxima ($m\mu$) (petroleum B.P. 70–80°)		Pigment present
pseudo-α-Carotene	Upper zone	484	452	β-Carotene
	Lower zone	477	446	*pseudo*-α-Carotene
α-Carotene	Upper zone	477	447	α-Carotene
	Lower zone	470	441	"Neocarotene" (*vide infra*)

This property clearly differentiates *pseudo*-α-carotene from α-carotene, hence we conclude the former to be a new pigment and not the racemic form of α-carotene.

By analogy with the known structural formula of α-carotene [Karrer, Morf & Walker, 1933] (I)



I.

it is probable that *pseudo*-α-carotene has a similar structure. From its method of preparation and properties it is to be inferred that the mechanism of formation is a displacement of one terminal double bond out of the conjugated system of β-carotene (II F). As this is more likely to be displaced to the 3′ 4′ position than to the 4′ 5′ or 5′ 6′ positions the resulting formula is probably the same as that of α-carotene (cf. Formula I). The closely similar properties (without actual identity) of *pseudo*-α-carotene and α-carotene make it probable that the difference is due to a rearrangement of the double bonds (leaving a group of ten conjugated) or to geometrical isomerism. The final decision on this point must await further work.

The effect of other adsorbents on β-carotene.

Having found that adsorption on alumina results in the isomerization of β-carotene, other adsorbents commonly used for the separation of carotenoids were tested for this property. Thus, calcium hydroxide, which has been used successfully for the separation of α-carotene from β-carotene [Karrer, Walker, *et al.* 1933] and the purification of the former [Karrer & Walker, 1933], has been found to convert β-carotene into *pseudo*-α-carotene exactly as does alumina, but it gives a smaller yield each time.

Magnesium oxide which has also been used [Strain, 1934, 1, 2] for the separation of α- and β-carotenes was similarly tested by repeatedly adsorbing β-carotene, in light petroleum solution, upon it. The β-carotene showed no evidence of separation into two zones even after four adsorptions. Furthermore, if a mixture of β-carotene and *pseudo*-α-carotene prepared by repeated adsorption of β-carotene on either alumina or calcium hydroxide is adsorbed on magnesium oxide the chromatogram shows no evidence of heterogeneity. There is reason to believe, however, that even if it does not produce any separable *pseudo*-α-carotene from β-carotene as do the other two adsorbents, after repeated adsorption on magnesium oxide, β-carotene begins to show displacement of its absorption bands towards shorter wave-lengths. Conversely, adsorption of *pseudo*-α-carotene displaces the maxima back again to wave-lengths not quite as long as those of β-carotene. The following data are typical.

Adsorption of pigments on magnesium oxide.

	Absorption maxima in petroleum (B.P. 70–80°) (mμ)	
Pure β-carotene	483	452
Pure β-carotene, after 1 adsorption	480	450
Pure β-carotene, after 2 adsorptions	479	449
pseudo-α-Carotene	476	446
pseudo-α-Carotene, after adsorption	479	448
"Neocarotene" (by adsorption on alumina)	470	441
"Neocarotene" (by adsorption on MgO)	472	443

We believe these differences in the positions of the absorption maxima to be significant but they are approaching the limits of detection by the method of photographic or visual absorption spectrophotometry unless high dispersion spectrographs are used.

Adsorption experiments with α-carotene.

We have carried out a few preliminary experiments on the effect of chromatographic adsorption on α-carotene (pure crystalline material *ex* palm oil; analytical data in Table I). Like β-carotene this material also suffers change on adsorption on alumina or calcium hydroxide and separates into two zones of pigment which, after elution, exhibit absorption maxima as follows:

	Carbon disulphide mμ		Petroleum (B.P. 70–80°) mμ	
Upper zone	507	477	477	477·5
Lower zone	501	470	470	441

The lower zone clearly contains a pigment distinctly different from either β- or α-carotene and which we propose to call *neocarotene*. The absorption maxima characteristic of this pigment are closely comparable with those reported for a yellow substance, occurring in small quantity at the bottom of the column, when α- and β-carotene mixtures are adsorbed on calcium hydroxide [Karrer & Walker, 1933]. It is now clear that this material must have been formed from α-carotene during the actual adsorption. α-Carotene is readily changed into neocarotene by adsorption on magnesium oxide (contrast β-carotene on this adsorbent).

Pending the results of a fuller examination of the properties of neocarotene which we are now conducting discussion of its probable structure is necessarily only conjecture, but the absorption spectrum, identical with that of taraxanthin [Kuhn & Lederer, 1931, 1], violaxanthin [Kuhn & Winterstein, 1931; Karrer *et al.* 1933], and α-oxycarotene [Karrer *et al.* 1934], suggests a straight chain of nine conjugated double bonds as the chromophoric grouping. From its method of preparation from α-carotene—strictly analogous with the preparation of *pseudo*-α-carotene from β-carotene—and from the light absorption data it is probable that it has the structure of α-carotene (Formula I) but with the β-ionone double bond displaced, probably into the 3, 4 position, thus leaving an α-ionone ring at each end and nine double bonds in conjugation. If this is correct neither half of the molecule will contain the half β-carotene structure essential for growth-promoting (vitamin A) activity, and the substance should therefore be biologically inactive, a forecast which we shall test when the material is available.

In considering the possible causes of the observed fact that when apparently pure β-carotene is repeatedly adsorbed on alumina it separates into two zones of pigment having different absorption spectra there appeared to be two possible explanations:

(*a*) that the original β-carotene contained *pseudo*-α-carotene as an impurity or,

(*b*) that the *pseudo*-α-carotene was formed from β-carotene.

The impurity hypothesis is definitely precluded by the fact that the carotene fractions remaining after one or more yields of *pseudo*-α-carotene had been removed were identical with each other, and with the original β-carotene. The following experimental results illustrate the point.

Fraction	M.P. (° C.)	Absorption bands petroleum (B.P. 70–80°) (mμ)		C (%)	H (%)
Original β-carotene	182	484	452	89·5	10·1
After one adsorption (no separation)	—	484	452	—	—
After two adsorptions:					
Upper zone	183	484	452	88·8	11·0
Lower zone	166	477	446	89·3	10·0
Readsorption of upper pigment only:					
Upper zone	—	483	452	—	—
Lower zone	—	476	447	—	—

There is thus no doubt that *pseudo*-α-carotene is not present as an impurity but is produced directly from β-carotene.

A further interesting point about this isomerization of both α- and β-carotenes is that it appears to be reversible, for whereas by separating the pigment formed by adsorption and readsorbing the unchanged pigment several times it can be wholly converted into the corresponding isomeride, if either of the new pigments is readsorbed on alumina or calcium hydroxide it is reconverted largely into what is apparently its parent substance. Thus we have converted β-carotene into *pseudo*-α-carotene and then back again. The properties of the resulting pigment are very similar to those of β-carotene.

Reversal of isomerization by adsorption.

Sample	Source	Adsorption on	M.P. °C.	Absorption bands petroleum (B.P. 70–80) mμ	
β-Carotene	Grass	—	182	484	452
pseudo-α-Carotene	β-Carotene	Alumina	166	477	446
β-carotene?	*pseudo*-α-Carotene	Alumina	175	480	452

Despite the fact that the process β-carotene $\rightleftharpoons$ *pseudo*-α-carotene will proceed in either direction on adsorption although not to completion, *pseudo*-α-carotene once formed does not apparently revert spontaneously to β-carotene even when kept for several months in light petroleum solution (in the absence of oxygen).

The basic or amphoteric nature of the adsorbents so far examined is probably a significant factor in producing these changes which are almost certainly due to displacements of one double bond out of the respective conjugated systems. It is clear however that the ease of this displacement is affected by the presence of impurities. Thus the carotene fractions of butter or blood serum, even after removal of sterols by freezing, are separated readily into two zones of pigment by a single adsorption on alumina [Gillam & El Ridi, 1935, 1], whilst with pure crystalline β-carotene it requires much longer washing, or more usually elution followed by another adsorption, to bring about the isomerization.

The possibility of these phenomena being due to oxidation is precluded by the following considerations.

(*a*) β-Carotene can be converted into *pseudo*-α-carotene by adsorption even when oxygen is rigorously excluded and when the adsorbent has been previously ignited in a stream of nitrogen to remove some or all of the adsorbed oxygen.

(*b*) The analytical data show that *pseudo*-α-carotene contains no oxygen (or only a trace due to autoxidation).

(*c*) If *pseudo*-α-carotene were an oxidized carotene derivative it would be adsorbed above β-carotene in the adsorption columns and not below it.

In view of the observation that certain adsorbents can isomerize α- and β-carotenes during the actual process of chromatographic analysis the technique as a whole must be more critically regarded. Originally due to Tswett [1906] this method of separation of pigmented substances by adsorption on suitable powders has been applied most successfully to the study of carotenoid pigments, notably by Kuhn and his collaborators [Kuhn & Brockmann, 1932, 2: Winterstein, 1933; Zechmeister, 1934]. Whilst there can be no doubt that in the vast majority of cases of the application of the method to carotenoid problems the separations obtained are genuine, substances undergoing adsorption are sometimes changed in the process, e.g., the formation of a red material by adsorption of vitamin A concentrates on alumina or calcium hydroxide [Castle *et al.* 1933].

The isomerization of α- and β-carotenes by adsorption on alumina, described above, is thus another exceptional example where the analytical process itself affects the substances which it is designed only to separate. In view of these cases it is important to realize that, following the usual procedure of chromatographic analysis, spurious separations can occur even if only rarely.

EXPERIMENTAL.

Preparation of pseudo-*α-carotene.* 200 mg. of pure β-carotene (*ex* cocksfoot grass; M.P. 180–182°; rotation, nil [Pollard, 1936]), made available through the kindness of Prof. A. C. Chibnall and Dr A. Pollard, were dissolved in light petroleum (B.P. 70–80°) and poured through a 50 × 6 cm. column of alumina (Brockmann's standardized *ex* Merck, diluted 1:3 with inactive alumina) and washed down with light petroleum (with or without the addition of benzene). The first adsorption, as a rule, did not show appreciable separation, but after elution with light petroleum-alcohol, followed by water washing, drying and another adsorption, a clear separation into two zones was usually obtained. By continued washing the lower zone was washed out of the column and collected separately. The unchanged β-carotene was eluted, recovered and readsorbed as before (4–5 times) until changed completely into the *pseudo*-α-form. In this way 200 mg. of β-carotene produces about 80–100 mg. of *pseudo*-α-carotene in some 4 litres of solvent, the remainder of the β-carotene being lost by oxidation, incomplete elution etc. Concentration of the solution by evaporation under reduced pressure in nitrogen with subsequent filtration, to remove alumina, and cooling, gave a crop of crystals which was further purified by recrystallization from light petroleum. Adsorption methods could not be used on account of the reversion to β-carotene.

ANALYSES. Micro-analyses (Pregl's method) gave the following results:

pseudo-α-*Carotene*. (1) Found: C, 89·3; H, 10·0%; $C_{40}H_{56}$ requires C, 89·5; H, 10·45%. (2) Found: C, 89·2; H, 9·9%.

β-Carotene. Found: C, 89·48; H, 10·1%.

α-Carotene. Found: C, 89·0; H, 10·6%.

ROTATIONS. Determinations carried out in red light (Pointolite lamp and Wratten 29 red filter giving only rays near the cadmium line at 6438 A.U.).

α-Carotene. 10 cm. 0·105% in benzene $+0·396°$; $[\alpha]_{Cd} = +377°$.

pseudo-α-*Carotene*. 10 cm. 0·15% in benzene $+0·002°$ $[\alpha]_{Cd} = +1·3° \pm 5°$.

HYDROGENATION. Microhydrogenations, for which we are indebted to Mr H. Jackson, gave the following results: Solvent, decalin-acetic acid mixture; Catalyst-PtO_2 (for details of method cf. Jackson & Jones [1936]).

pseudo-α-*Carotene*. 2·522 mg. absorb 1·215 ml. of H_2 at 766·5 mm. and 21·2°; double bonds, 10·9.

The *pseudo*-α-carotene for hydrogenation was specially prepared using light petroleum and benzene free from thiophen. When less pure solvents were used the pigment contained sufficient sulphur to poison the catalyst and stop the hydrogenation either wholly or in part.

β-Carotene ex *cocksfoot grass*. 1·582 mg. absorb 0·780 ml. of H_2 at 754·5 mm. and 17·5°; double bonds, 11·0.

α-Carotene ex *Palm oil*. 1·470 mg. absorb 0·725 ml. of H_2 at 15·6° and 755 mm.; double bonds, 11·0.

ABSORPTION SPECTRA of the pigments were determined on a Hilger-Nutting visual Spectrophotometer, whilst objective confirmation was obtained photographically on a Hilger E_3 quartz spectrograph fitted with a Spekker photometer.

SUMMARY.

Following up previous work on the carotene of butter where it was found that adsorption on alumina or calcium hydroxide separates the pigment into two parts which simulate α- and β-carotene, respectively, it has now been found that what was apparently α-carotene is largely made up of a new pigment, spectroscopically identical with it.

The new pigment—*pseudo*-α-carotene—has been prepared by repeated chromatographic adsorption of β-carotene on alumina. It has M.P. 166°, is isomeric with β-carotene, and on readsorption is reconverted partially into this compound. Although spectroscopically identical with α-carotene it differs in having no rotation. It further differs in adsorption properties and hence is probably not a racemic form of α-carotene. It is suggested that the mechanism of formation is a displacement of a terminal double bond out of the conjugated system by the adsorption process.

Preliminary experiments with α-carotene indicate that this also can be changed by adsorption into another carotenoid ("neocarotene") the absorption maxima of which are displaced even further towards the violet from those of α-carotene. Its properties are being further examined.

It is pointed out that in using the chromatographic method for the separation of carotenoids care must be taken to differentiate between genuine separations and transformations brought about by the process itself.

We wish to acknowledge our indebtedness to Prof. I. M. Heilbron for his interest and advice, to Messrs Lever Bros., Ltd. (Dept. CTD/F) for a generous supply of palm oil unsaponifiable matter, and to Mr B. Lythgoe for a sample of α-carotene. Our thanks are also due to Messrs Imperial Chemical Industries for a grant, and to the University of Cairo for facilities enabling one of us (M. S. El R.) to participate in the work.

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CCXLIV. THE NATURE OF PARANUCLEIN.

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(*Received 8 July 1936.*)

The traditional misnomer "paranuclein" has been somewhat indiscriminately applied to a variety of phosphorus-containing degradation products of caseinogen; here paranuclein will refer specifically to the material deposited during the digestion of caseinogen by pepsin.

The experiments of the earlier workers (summarized by Rimington & Kay [1926]) showed that the material varied considerably, according to the experimental conditions, both in total amount and in its content of the original caseinogen-phosphorus. To explain these variations in phosphorus content, Robertson [1907], in work in which he claimed to have synthesized paranuclein from lower degradation products of caseinogen, expressed the opinion on somewhat slight evidence that paranuclein must be a mixture of at least two substances. Thereafter for a time the subject lapsed until Rimington & Kay, in their well-known studies on caseinogen, investigated also the derived paranuclein, and definitely confirmed its resistance to pepsin and its ready degradation by trypsin and by 1 % NaOH.

Holter *et al.* [1932] analysed paranuclein or "phosphorpepton" with regard to its identity as a single compound, and found a N/P ratio[1] of 8·3 to 8·7 among some half-dozen specimens, the divergence being assumed to be within the limits of experimental error. On prolonged digestion, they found that the N/P ratio fell to 7·1. Stirling & Wishart [1932] investigated the organic phosphorus produced on peptic digestion of caseinogen, using the technique of precipitation by trichloroacetic acid, and found a fairly constant N/P ratio for the insoluble residues of 12·8. This work, however, is difficult to correlate with the previous since the acid-insoluble residues and paranuclein are clearly not identical. Breese-Jones & Gersdorff [1934] analysed various fractions produced in the peptic digestion of caseinogen, of which paranuclein was one: as prepared by them, it had a N/P ratio of 6, and showed several interesting changes in amino-acid composition from the original caseinogen, markedly the absence of cystine.

Certain work on the other phosphorus derivatives of caseinogen is also of interest. Salkowski [1901] prepared a "paranucleic acid" by precipitation of the digest liquor with ferric ammonium sulphate, which as finally purified had a N/P ratio of 3·1, and was precipitated by tannic and phosphotungstic acids though but slightly by trichloroacetic acid. Dietrich [1909], following up some work by Reh, obtained several phosphorus-containing peptides from peptic digests, which had N/P ratios varying from 0·45 to 1·75. A well-defined phosphopeptone of basic character was prepared by Rimington [1927] from tryptic digestion of caseinogen; it had a N/P ratio of 1·44 and gave no precipitate with phosphotungstic or trichloroacetic acid. Posternak [1927] has described a phosphopeptone of N/P ratio 2; it appears to differ from any others described. Utkin [1936], in an investigation of the rate of liberation of soluble phosphorus from caseinogen by pepsin, finds evidence suggestive of two different phases; the first concerns the liberation of 19–20 % of the material as a compound of N/P ratio 7, recalling paranuclein; the second phase is characterized by liberation of material of N/P ratio 3·5, which he considers may be related to Salkowski's "paranucleic acid".

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[1] The N/P ratios mentioned here are in every case those of absolute, not atomic proportion, calculated where necessary from the original data.

From the above it is obvious that there is no unanimity of opinion as to whether paranuclein is an individual substance, or what role it may play in the degradation of caseinogen. The following work was undertaken in the hope that time-course studies of its hydrolysis by enzymes and by alkali might throw some light on its nature.

EXPERIMENTAL PROCEDURE.

Preparation of paranuclein. 10 g. caseinogen (*nach Hammarsten*, N/P:17·25) were rubbed in a mortar with water until a fairly smooth paste was obtained. *N* HCl, 1·2 ml. per g. caseinogen, was then added gradually with constant stirring. The mixture was made up to 300 ml. and allowed to stand overnight in the refrigerator. At first, this solution was centrifuged and the supernatant liquor decanted; the residues were dissolved in a further 150 ml. water, filtered, and the filtrate added to the original liquor. Later, the more dilute solution was made up directly, it being found that any undissolved material disappeared during digestion before the paranuclein began to precipitate.

2 g. pepsin (B.D.H.), dissolved in 100 ml. water, were added to the above caseinogen solution and incubated at 37·5°. Simple measurements showed that the point of maximum precipitation occurred at the end of one hour. This was found to be fairly coincident with the point at which the precipitate began to settle into flakes [cf. Holter *et al.* 1932], and the latter was used as the end-point of the reaction. The precipitate was now centrifuged off, washed once with water, recentrifuged, and dried *in vacuo*. The yield is just over 20%; the N/P ratio has been found to vary from 7·1 to 8·8.

Time-course of hydrolysis.

1 % NaOH. A weighed amount of paranuclein was suspended in water, immersed in the thermostat, and at a noted time *N* NaOH was added in the requisite quantity to give a final concentration of 1%. The paranuclein dissolved immediately. 5 ml. samples were removed at noted intervals and treated with an equal volume of trichloroacetic acid. The filtrate after precipitation and the residue of the original solution were then subjected to analysis for determination of acid-soluble and total N and P respectively.

That no loss of N as ammonia occurred during incubation was established in the first experiment by the identity of values obtained for total N at the beginning and end of the incubation.

Trypsin. A weighed amount of paranuclein (about 0·5 g.) was suspended in 20 ml. water and immersed in the thermostat. To it were added at a noted time 10 ml. of trypsin solution (Difco) and immediately sufficient *N*/10 NaOH to give a faint pink with phenolphthalein. The mixture was then diluted to 50 ml. Samples of 5 ml. were removed at noted times, precipitated by trichloroacetic acid and analysed. Noted amounts of *N*/10 NaOH were added during the incubation to maintain the *p*H at 8·5; it was felt that this procedure was preferable to the use of a buffer since salt effects might complicate the reaction.

Pepsin. Preliminary experiments with dilute solutions of pepsin, similar to those with trypsin, indicated that hydrolysis was extremely slow. With high pepsin concentrations measurable hydrolysis occurred, but the large values of the N and P of the controls increased the experimental error considerably. The data recorded for this enzyme are therefore limited to one experiment in which the following method was used.

5 g. pepsin (B.D.H.) were dissolved in 100 ml. *N*/10 HCl; 50 ml. of this solution were added to 1 g. paranuclein, the remaining 50 ml. served as control. Samples were taken only (*a*) as soon as possible after the addition of the enzyme and (*b*) after 20 hours' incubation; they were precipitated and analysed in the usual way.

Papain. The literature is rather contradictory with regard to the optimum *p*H for papain action. Plimmer & Bayliss [1905] used it in slightly acid or neutral medium on caseinogen. Willstätter & Grassmann [1924; 1926, 1, 2] found an optimum *p*H for various proteins corresponding in each case to the isoelectric point. The presence of certain salts has, however, an important effect [Ringer, 1935]. Further complications arise when the use of activators is considered.

Preliminary experiments with citrate-NaOH buffers at different *p*H values showed that maximum activity was obtained at *p*H 6·7, and that the enzyme was sufficiently active for the purpose without the use of an activator. One experiment with the addition of an activator was however carried out for comparison. The final procedure was as follows.

Approximately 0·5 g. paranuclein was suspended in 30 ml. water and 15 ml. citrate buffer *p*H 6·7 were added. After 5 min. immersion in the thermostat, 5 ml. enzyme extract were added; the extract was prepared by allowing 1 g. papain powder (B.D.H.) to stand overnight at 0° with 10 ml. water, centrifuging and decanting, the liquor being diluted as required to give final concentrations of approx. 1, 0·2 and 0·1 % papain.

The experiment with 0·1 % papain was repeated with an enzyme extract which had been activated by cysteine hydrochloride, as described by Purr [1935].

All incubations were carried out at 37·5 ± 0·1° and the trichloroacetic acid was invariably used in 10 % concentration. Nitrogen was estimated by micro-Kjeldahl and phosphorus by the Fiske-Subbarow method throughout. The values of N and P given in the tables have been corrected for the N and P of the enzyme solution present. It may be remarked incidentally that whilst pepsin-P was entirely acid-soluble, papain-P was partially so and trypsin-P was entirely non-acid-soluble. In all cases, the N was partly acid-soluble.

Three blank experiments were also carried out—two in dilute NaOH at *p*H 8 in the absence of trypsin, and one in citrate buffer of *p*H 6·7 in the absence of papain.

RESULTS.

Since the results of the blank experiments play a large part in the interpretation of the enzymic hydrolyses, they are given first in Table I, Exps. 1, 2, 3.

Table I.

	*p*H 8				*p*H 6·7 (citrate)	
Exp.	1		2		3	
Total N/5 ml. ...	9·26 mg.		6·30 mg.		6·59 mg.	
Total P/5 ml. ...	0·842 mg.		0·786 mg.		0·842 mg.	
N/P	8·6		8·0		7·8	
	% acid-soluble		% acid-soluble		% acid-soluble	
Time	N	P	N	P	N	P
5 min.	—	—	40·5	35·6	39·5	39·0
15	39·3	35·6	—	—	—	—
30	39·7	35·9	41·1	36·0	42·9	40·4
1 hr.	40·0	36·9	41·6	36·5	46·6	42·5
2	—	—	—	—	48·1	44·9
3	41·5	37·1	42·2	36·5	—	—
4	—	—	—	—	49·2	46·0
7	—	—	—	—	51·1	48·9

From them, the surprising fact emerges that about 40 % N and 35 % P are already soluble in trichloroacetic acid. No appreciable further hydrolysis occurs at *p*H 8; at *p*H 6·7 there is a slight further hydrolysis.

Omitting further consideration of this point for the moment, it is obvious that enzymic digestion of paranuclein as followed by the action of trichloroacetic acid will show only the rate of digestion of the insoluble fraction of the paranuclein. This fact is, indeed, confirmed by the time curves of enzymic hydrolyses given below, since the best-fitting curves to the gross amounts of acid-soluble N and P (with the exception of pepsin) cut the ordinate at values approximating to those obtained in the blank experiment.

Digestion with 1 °/₀ NaOH.

The individual results of this series are shown in Table II and Exp. 4 is depicted graphically in Fig. 1 (upper pair of curves).

Table II.

Exp.	4		5		6	
Total N/5 ml. ...	6·04 mg.		6·67 mg.		6·13 mg.	
Total P/5 ml. ...	0·746 mg.		0·777 mg.		0·700 mg.	
N/P	8·1		8·6		8·8	
	% acid-soluble		% acid-soluble		% acid-soluble	
Time	N	P	N	P	N	P
2 min.	53·3	37·5	43·9	30·3	47·8	30·3
15	63·9	50·0	51·7	34·1	56·9	41·3
30	66·7	55·6	56·1	41·3	59·7	51·4
1 hr.	72·3	70·5	59·5	49·0	—	—
1·75	79·1	83·4	64·8	63·5	—	—
2·5	80·0	91·6	—	—	69·3	62·3
4·5	—	—	76·8	78·3	—	—
*7	80·0	99·9	79·3	83·4	75·5	75·4
24	81·0	101·6	—	—	77·6	79·6
72	82·8	103·2	—	—	83·4	87·6

* 6 hr. in the case of Exp. 1.

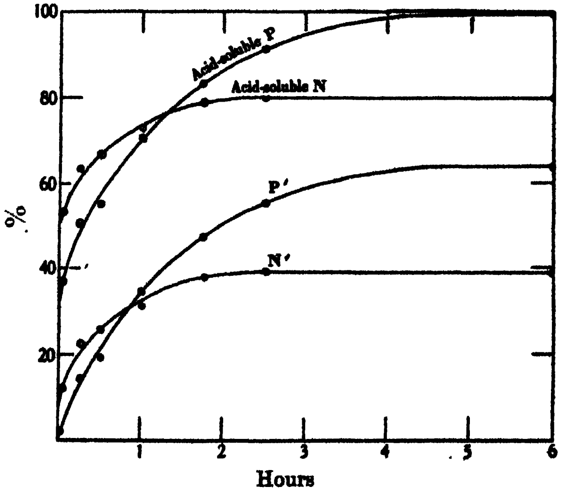

Fig. 1. Action of 1 % NaOH. N′ and P′ represent the hydrolysis of the originally acid-insoluble material.

Digestion by trypsin.

The results with various enzyme concentrations are shown in Table III and Exp. 9 in Fig. 2 (upper pair of curves).

Table III.

Exp.	7		8		9		10		11	
Final trypsin conc. ...	0·1 %		0·02 %		0·02 %		0·004 %		0·004 %	
Total N/5 ml.	8·79 mg.		7·36 mg.		7·23 mg.		4·76 mg.		10·32 mg.	
Total P/5 ml.	1·14 mg.		0·993 mg.		0·937 mg.		0·624 mg.		1·197 mg.	
N/P	7·7		7·4		7·7		7·6		8·6	
	% acid-soluble		% acid-soluble		% acid-soluble		% acid-soluble		% acid-soluble	
Time	N	P	N	P	N	P	N	P	N	P
5 min.	81·3	83·3	53·1	39·2	45·1	42·3	49·0	43·8	52·2	28·0
15	92·5	100·9	70·3	69·6	77·6	70·8	61·1	55·5	60·6	33·1
30	98·3	102·7	78·5	90·6	82·9	90·3	75·2	69·1	67·7	42·4
45	—	—	83·4	98·0	90·2	96·6	84·1	87·2	71·8	52·5
1·25 hr.	98·5	103·5	93·9	97·8	97·5	99·0	87·6	94·1	78·1	62·3
2	—	—	98·0	96·6	—	—	91·4	96·5	—	—
3	—	—	99·6	99·9	100·0	99·8	98·1	102·4	87·3	77·5

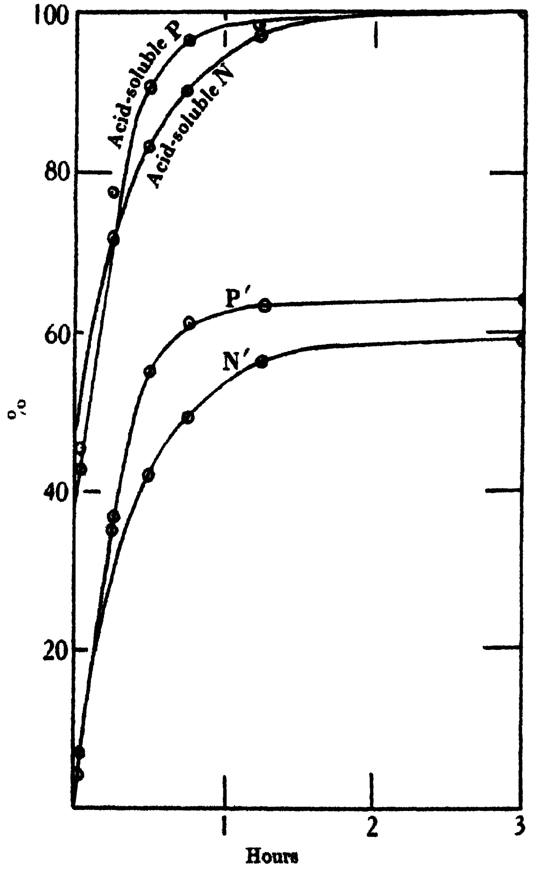

Fig. 2. Action of 0·02 % trypsin. N′ and P′ represent the hydrolysis of the originally acid-insoluble material.

Digestion by pepsin.

The results of the single experiment with pepsin are given in Table IV.

Table IV.

Exp. 12

Total N/5 ml. 13·4 mg. Total P/5 ml. 1·74 mg. N/P, 7·7

Time	% acid-sol. N	% acid-sol. P
2 min.	19·4	20·2
20 hr.	77·6	71·3

Digestion by papain.

The effects of three different concentrations of papain are shown in Table V and Exp. 15 in Fig. 3 (upper pair of curves). Exp. 16 of this group was analogous to Exp. 15 but before incubation the papain was activated by cysteine HCl.

Table V.

Exp.	13		14		15		16	
Final papain concentration ...	1·0%		0·2%		0·1%		0·1%	
Total N/5 ml. ...	6·54 mg.		6·18 mg.		6·47 mg.		6·05 mg.	
Total P/5 ml. ...	0·905 mg.		0·806 mg.		0·860 mg.		0·811 mg.	
N/P	7·23		7·7		7·5		7·5	
	% acid-soluble		% acid-soluble		% acid-soluble		% acid-soluble	
Time	N	P	N	P	N	P	N	P
1 min.	45·4	41·8	45·0	39·0	45·8	39·2	49·6	51·1
15	86·2	75·4	56·1	46·2	51·3	45·8	76·0	82·6
30	94·2	85·3	70·7	62·4	55·0	52·7	84·3	93·0
45	95·3	86·5	—	—	—	—	—	—
1 hr.	96·2	89·1	81·2	74·8	59·7	59·1	87·1	97·9
2	98·9	90·4	86·2	79·3	66·3	66·0	90·9	99·6
3	101·5	93·5	91·3	83·8	—	—	—	—
4	—	—	98·1	86·9	78·2	80·0	96·4	101·9
7	—	—	—	—	90·0	90·7	100·8	101·6

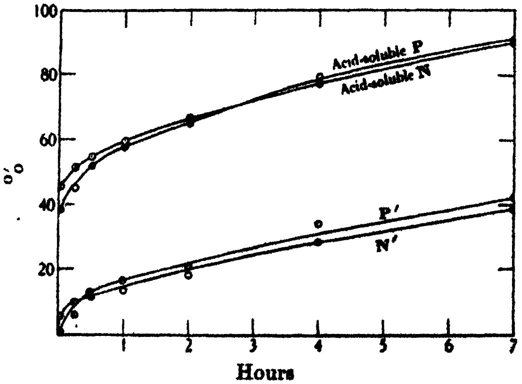

Fig. 3. Action of 0·1 % papain. N′ and P′ represent the hydrolysis of the originally acid-soluble material.

From these results it will be seen that the preformed acid-soluble material is present to approximately the same extent in all but Exp. 12 with pepsin. To arrive at the changes due to enzymic hydrolysis, it is therefore justifiable to deduct from the gross values the values of the blank experiments given in Table I. The nett values so obtained are illustrated in the lower pair of curves in each graph.

By this deduction, the crossing of the curves in the tryptic hydrolysis is entirely removed; with papain the crossing is much less marked and may be due to experimental error involved in estimating the small quantities of acid-soluble N and P in the early stages; with 1 % NaOH however the crossing involves larger values than are probably explicable in this way. In general, with both trypsin and 1 % NaOH, the P is more rapidly liberated in acid-soluble condition than the N. The experiments with 1 % NaOH disclose the existence of a residue containing some 20 % N and no P which is resistant to the action of alkali.

With peptic hydrolysis, there is again evidence from the early samples of the existence in the solution of preformed acid-soluble N and P, but in much smaller amounts than in the trypsin or papain hydrolysis—about 20 %. At first, this was assumed to be due to the acid reaction but, as will be shown below, the behaviour of paranuclein in the presence of pepsin is peculiar. Since, however, the amounts of preformed N and P are practically identical, their deduction would not influence the relative positions of the N and P curves, and it may be definitely stated that pepsin differs from the other hydrolytic agents in liberating acid-soluble N more quickly than P.

With non-activated papain, there is little difference in the relative rates at which acid-soluble N and P are liberated. With the activated papain (Exp. 16), the rates of hydrolysis of both N and P are accelerated, particularly that of P. It is hoped to investigate this acceleration further.

The most interesting disclosure is, however, the presence of a preformed acid-soluble fraction in the paranuclein, and we returned to a further investigation of its nature. The caseinogen itself was shown, as expected, to have no acid-soluble fraction, the results obtained being of the order of 0·002 % N and no detectable P. Experimentation was then directed towards discovering whether the two fractions of the paranuclein could be separated by simple solution at different *p*H values.

Some paranuclein was suspended in water in the thermostat and left for 30 min. to come to equilibrium. The *p*H of this suspension was 4·2. Samples of 5 ml. were then brought to different *p*H values by addition of HCl or NaOH as required, the *p*H being controlled by indicators. After standing a further 30 min. in the thermostat, the samples were filtered without further treatment and the N and P of the filtrate determined (Exp. 17).

Another series was treated in similar fashion, but before filtration 5 ml. trichloroacetic acid were added to each sample (Exp. 18).

Table VI.

Exp.	17		18 (Precipitated by trichloroacetic acid)	
Total N/5 ml. ...	5·89 mg.		7·36 mg.	
Total P/5 ml. ...	0·806 mg.		0·903 mg.	
N/P	7·3		8·2	
	Filtrate		Filtrate	
*p*H value	% N	% P	% N	% P
2·0	49·1	42·4	51·9	48·9
3·5	44·5	41·3	45·9	44·5
4·2	44·1	42·7	46·1	44·0
5·5	70·3	68·0	47·8	43·6
7·0	87·9	88·8	45·6	43·4
8·5	94·4	93·7	—	—

As the results show, separation of the paranuclein can be produced by mere adjustment of *p*H. The trichloroacetic acid-insoluble fraction of paranuclein is also insoluble in aqueous solution at *p*H 4·2 and below; above this *p*H the solubility of the fraction rapidly increases until, at *p*H 8·5, the paranuclein is almost entirely soluble.

Although unconnected with the main argument, comment may be made on the fact that in Exp. 17 there is evidence of slight hydrolysis at pH 2. More important, there exists even at this pH a soluble fraction containing almost half of the N and P. Why, then, is the paranuclein precipitated at this pH during its formation by peptic digestion? The original digestion mixture from which the paranuclein was prepared differed from that in Exp. 17 only in the presence of undigested caseinogen, degradation products other than paranuclein and of pepsin. The influence of these materials was tested by precipitating paranuclein with trichloroacetic acid from solutions containing in addition (*a*) caseinogen and (*b*) pepsin. The final concentration of each was 1 %. There was no lowering in (*a*) of the acid-soluble material, but (*b*) showed a fall in soluble N to 25 % in one case and 33·6 % in another, with comparable P values. It may be recalled also that in the original time-course experiments on peptic hydrolysis, in which 5 % pepsin was used, the immediately acid-soluble N and P were approximately 20 %. Apparently the mere presence of the pepsin preparation used influences the acid-solubility of the paranuclein. To complete the analogy with Exp. 17, another test was made on the effect of pepsin on the solubility of paranuclein as shown by simple filtration, no trichloroacetic acid being used. The soluble N amounted to 37 %, the P to 30 %. It is hoped to investigate this effect further.

Returning to the question of the dual nature of paranuclein, from its physical behaviour the existence of two loosely bound fractions seems undeniable and attention was turned towards a possible chemical differentiation of these fractions.

A suspension of paranuclein was filtered, and to the filtrate and insoluble residue the tests given below were applied; the two fractions were also combined with iodine according to the method of Blum & Strauss [1920] by solution in sodium carbonate and addition of a solution of iodine in potassium iodide.

The results of the qualitative tests and the percentages of iodine bound are shown in Table VII.

Table VII.

	Residue	Filtrate
Biuret	Violet	Violet
Xanthoproteic	+	+
Labile S	+	–
Millon	+	Very faint
Glyoxylic	+	Very faint
Iodine %	10·30	26·19

These results suggest a deficiency in the soluble material of tyrosine and tryptophan, and an absence of labile S. The power of combination with iodine also clearly distinguishes the two fractions.

DISCUSSION.

The crossing of the original time-course curves for acid-soluble N and P is obviously mainly due to the fact that, in a solution of paranuclein, there exist already in acid-soluble form considerable fractions of the N and P, approximately 5 % more of the former than the latter. The deduction of this "preformed" soluble N and P in all cases diminishes, and in some abolishes, the crossing of the original curves. It is possible that the slight degree of crossing which remains is in some manner due to experimental error.

After correction of the curves by this deduction, they show that, in hydrolyses by alkali and trypsin, the P is liberated in acid-soluble form more rapidly than the N. With pepsin, the reverse holds, so that its mode of attack on the substrate must be different. Similar findings were obtained by Stirling & Wishart [1932] for the peptic and tryptic digestions of caseinogen. Furthermore, digestion of paranuclein by trypsin is very rapid, that by pepsin very slow. Papain takes an intermediate place both in regard to activity and the relative rates of N and P liberation; the acceleration on activation is rather strikingly greater for P liberation than for N. The action of NaOH differs however from that of the enzymes in leaving unattacked a fraction of the paranuclein containing some 20 % of the original N and no P.

The most important disclosure from these time-course experiments was the indication they gave of the existence in paranuclein of two fractions: and the question of the constancy of composition of this substance is reopened. There seems little doubt from a study of the literature that the total amount of paranuclein produced is dependent on the experimental conditions, though there is equally little doubt that under similar conditions a fairly constant product is obtained. Linderstrøm-Lang and his co-workers find their paranuclein to contain 2/3 to 3/4 of the total caseinogen phosphorus, and to have a N/P ratio of 8. Breese-Jones & Gersdorff's product contained about 1/3 of the caseinogen phosphorus and had a N/P ratio of 6. Our own experience is similar to that of Breese-Jones & Gersdorff with regard to phosphorus content, but the average N/P ratio of our preparations is in the neighbourhood of 8. It is quite possible that the lower ratio of their material is due to the repeated washing of the centrifuged precipitate, thus removing a certain amount of material of higher N/P ratio, that which we have found acid- and water-soluble.

The variations in phosphorus content do not invalidate the hypothesis that a single substance is being formed, and this hypothesis is supported by the comparative constancy of composition of the samples prepared by Linderstrom-Lang and co-workers from widely different preparations of caseinogen. On the other hand, the variations of composition found by the early workers led Robertson to suggest that paranuclein may be a complex of more than one substance, the components being precipitated together under the conditions of peptic digestion.

The present results seem to point definitely to the latter conclusion, or at least to the existence of two loosely bound fractions, since on mere solution at different *p*H values almost half of the paranuclein is not precipitated by trichloroacetic acid. Further, a quantitatively similar separation is effected by simple filtration of the suspension at acid *p*H values, and the fractions so obtained differ in their amino-acid composition and power of combination with iodine.

But whilst it is clear that, in the paranuclein as separated from the original peptic digest, there are these two fractions, the fact that one of them is soluble in water at *p*H 2 is very difficult to reconcile with its appearance in insoluble form during peptic digestion. The necessarily low solubility of the paranuclein as originally formed can be reproduced to a certain extent, however, by the addition of pepsin to solutions of the separated and dried material, and three possibilities suggest themselves. First, the soluble material is precipitated in combination with a component of the commercial pepsin used; the combination of a component of the enzyme with one of its degradation products is well within precedent. Second, the soluble material is liberated from the caseinogen in loose combination with the other, insoluble, fraction of the paranuclein; the very ready hydrolysis of this loose complex might then be reversed only by the synthetic action of pepsin, which would explain why the reversal obtained was never complete. Third, the paranuclein itself is a synthetic by-product of the original reaction; and we may refer again to the ready synthesis by Robertson [1907] of what he claimed was paranuclein.

With regard to the relation between paranuclein and the various other phosphorus-containing degradation products of caseinogen which have been described in the literature, nothing definite can yet be said. Presumably the rise in phosphorus content of paranuclein on prolonged peptic digestion noted by various workers is due to the loss of the fraction of higher N/P ratio which we have found water-soluble.

It appears an attractive hypothesis that paranuclein is formed during tryptic digestion of caseinogen but does not become evident because of the alkalinity of the medium, and that Rimington's phosphopeptone is one of its further degradation products. The present findings, however, and the previous ones of Stirling & Wishart [1932] indicate that the modes of attack of pepsin and trypsin on caseinogen are fundamentally different, and it becomes unlikely that paranuclein is formed by trypsin. At the same time, it cannot be denied that phosphopeptone may have analogues in peptic digestion; this is suggested in fact by Dietrich's finding of acid peptones of N/P ratio similar to that of phosphopeptone during peptic digestion.

Similarly with the "paranucleic acid" prepared by Salkowski [1901] and believed to have been obtained also by Utkin, the relation between these products and paranuclein will not be clarified until they have been prepared by digestion of paranuclein itself, or isolated from a caseinogen digest from which all paranuclein has been previously removed.

Summary

Time-course studies are reported of the rate at which the N and P of paranuclein are rendered soluble in trichloroacetic acid by the action of pepsin, trypsin, papain and 1 % NaOH. The commercial preparation of papain is intermediate in action between pepsin and trypsin; the fully activated form is different. The action of 1 % NaOH discloses the existence of a nucleus containing 20 % of the paranuclein N and no P, which is resistant to alkaline hydrolysis.

These time-course studies indicated in addition that paranuclein contains at least two fractions. This was confirmed by the finding of differences in physical behaviour, in amino-acid composition and in iodine-combining power of the two fractions.

To Prof. Wishart, I wish to record my gratitude for his unfailing interest and helpful advice during the course of the investigation. This work was performed during the tenure of the Barbour Scholarship of Glasgow University.

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CCXLV. OBSERVATIONS ON THE USE OF THE PHOSPHOTUNGSTIC ACID METHOD OF DETERMINING ASCORBIC ACID IN URINES WITH LOW ASCORBIC ACID CONTENT.

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(*Received 20 June 1936.*)

Since publication of the method for the determination of ascorbic acid in urine with phospho-18-tungstic acid [Medes, 1935], special difficulties attendant upon its use in urines of extremely low ascorbic acid content have been pointed out to the author by Dr J. C. Andrews and by Dr G. A. Harrison, for whose suggestions she would like to express her appreciation.

1. *Colour.* Urines containing as little as approximately 4×10^{-4} g. mol. ascorbic acid per 100 ml. develop a blue colour with phospho-18-tungstic acid of such intensity that the yellow colour of the urine does not usually interfere in the colorimetric determinations. When, however, the ascorbic acid content falls greatly below this figure, the urinary pigments may predominate to such an extent that a pale yellowish green colour results. The difficulty may be met by a number of devices.

(*a*) When the interference is slight, the colour of the standard may be slightly altered by the introduction of a few drops of one of the indicators which are yellow in acid solution, such as bromophenol blue, as suggested by Shinohara & Padis [1936]. This method necessitates a series of standards containing graded amounts of the indicator. When the colour is a greenish yellow, such large amounts of the indicator must be added that an appreciable error is introduced, and this method cannot be employed.

(*b*) A colour filter (Monochromat Wratten No. 75, Eastman Kodak Company) in the ocular of the colorimeter, employed by Dr Andrews, proved satisfactory in a series of tests made by the author.

(*c*) Backing the standard by urine, diluted similarly to that in the test solution. In the author's laboratory, the McClendon compensating cups for hydrogen ion determinations are employed in routine determinations made directly on urine. The only precaution to be observed is that the standard and unknown must be of approximately the same intensity; otherwise satisfactory compensation is not attained.

2. *Cloudiness of the urine.* In urines extremely low in ascorbic acid a faint cloudiness frequently develops when the uric acid reagent is added (Andrews and Harrison, private communications). No explanation is offered for this phenomenon. Neither the author nor the investigators mentioned above have observed its occurrence in urines containing more than a trace of the vitamin. According to Harrison, some of these specimens fail to clear on centrifuging. Accurate readings cannot be secured in the colorimeter, values as high as ten times those determined by the titrimetric method being obtained. The colorimetric results in Table I are from determinations made by J. C. and K. C. Andrews on uncentrifuged specimens.

Table I. *Comparison of the determination of ascorbic acid in 4 samples of urine with phospho-18-tungstic acid and by titration with dichlorophenolindophenol.*

Colorimetric method			Titration method		
Av. value g. mol. × 10^{-6} per 100 ml.	Av. deviation %	Max. deviation %	Av. value g. mol. × 10^{-6} per 100 ml.	Av. deviation %	Max. deviation %
4·92	14·0	21·0	5·15	10·9	17·4
12·7	9·3	16·2	14·6	9·7	21·2
21·7	2·6	4·4	27·3	7·0	15·4
39·7	1·5	2·5	37·9	5·1	9·3

In the author's laboratory 100 specimens were investigated, all those giving more than a faint green colour with the reagents being rejected. About half of those selected showed faint cloudiness and 25 with appreciable cloud were retained for further study. To each one the reagents were added, the specimen was transferred immediately to the centrifuge and centrifuged at highest speed for 15 min. while the colour was developing. In all cases a clear specimen was obtained.

3. *Time element in development of the blue colour with the uric acid reagent.* In the original description of the phospho-18-tungstic acid method it was stated that readings in the colorimeter should be taken after 20 min. In many urines, colour continues to develop even after this period. This is not the case with pure solutions of ascorbic acid. In other urines, the colour remains constant from about 15 min. after the reagents are added up to several hours. It was concluded, therefore, that this persistent development of colour was not due to the ascorbic acid, but to some unknown factor and that the reading taken immediately at the close of the 20-min. period represents most nearly the true ascorbic acid value.

It is not known to what factors this gradual increase of colour is due. Shinohara [1935] investigated the effect on the uric acid reagent of a number of the normal constituents of urine, and although he found that several of them caused a slow appearance of colour, none produced an appreciable effect within 20 min. when present in physiological amounts. The author has extended these experiments, using varying combinations of these and other constituents, to determine how far their effects may be additive, but failed to account for the reaction. It seems possible that the urinary pigments, which probably have some reducing properties, may be at least partially responsible, since the phenomenon occurs most frequently in highly coloured urines. The precaution of taking the colorimeter readings at the close of the 20-min. period ± 5 min. should therefore be observed for all urines.

Accuracy of the method in urines containing small amounts of ascorbic acid. From a group of urines from the hospital wards, 4 were selected for comparison of the two methods, colorimetric and titration with dichlorophenolindophenol. The concentrations of ascorbic acid ranged from about $4{\cdot}0 \times 10^{-5}$ g. mol. per 100 ml., or approximately matching the 2 ml. standard, to $5{\cdot}0 \times 10^{-6}$ g. mol. per 100 ml., with which a standard containing 0·5 ml. of 0·001 *M* ascorbic acid should be employed against 10 ml. of urine. Table I gives the average values obtained in a series of 5 consecutive determinations by each method, with the average and maximum deviations in each group, expressed in percentages of the mean.

When about 5×10^{-6} g. mol. ascorbic acid was present, the maximum deviation from the mean was about 20 % by the two methods, the average deviations being 14 and 11 % respectively. As the concentration of ascorbic acid was increased, the error of a single determination decreased much more rapidly by the colorimetric than by the titration method, until when about 4×10^{-5} g. mol. ascorbic acid was present, the maximum deviations from the mean became 2·5 and 9·3 % respectively.

A further test of the relative accuracy of the two methods was performed by adding increments of standardized ascorbic acid to the urines employed above and determining the percentage recovery. When 5 ml. of 0·001 *M* ascorbic acid were added to 100 ml. of the urines with lowest vitamin content, the average recovery ranged between 95 and 106 %, with average deviations approximately agreeing with those in the table for the corresponding urines. When 1 ml. of 0·01 *M* ascorbic acid was added to 100 ml. of the same urines, the recovery averaged 99 ± 5 % by the colorimetric and 104 ± 11 % by the titration method.

In other words, with extremely low concentrations of ascorbic acid the two methods are about equally accurate in the hands of the present investigator, and with concentrations of ascorbic acid which give rise to a blue colour with the phosphotungstic acid reagent, the colorimetric becomes the method of choice.

SUMMARY.

In using the phospho-18-tungstic acid method for the determination of ascorbic acid in urine, special precautions must be employed for urines with very low ascorbic acid content.

Suggestions are offered for compensation for the yellow colour developed when the urinary pigment interferes with comparison of standard and unknown.

The cloud which frequently develops in these urines may be removed by centrifuging.

Readings in the colorimeter should be taken within 20 ± 5 min.

In a series of tests on urines with low ascorbic acid content, the colorimetric and titration methods are of about equal accuracy. With urines of higher ascorbic acid content, the colorimetric method gives more highly accurate results.

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CCXLVI. EXPERIMENTS ON METABOLISM WITH *C. DIPHTHERIAE*. I.

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The preparation of diphtheria toxin on a large scale is, in spite of the numerous investigations which have been carried out on this subject, still a very uncertain operation. Without doubt the cause of this must be sought to a considerable extent in the fact that the usual culture media are of a very complicated nature and always contain besides the simple compounds such as sugars, inorganic salts etc., meat extract and one or other of the various kinds of peptone.

Most of the experiments on the dissimilative metabolism of *C. diphtheriae* mentioned in the literature have been carried out on media of much simpler composition.

It is a striking fact, however, that in all these experiments little or no attention is paid to the formation of toxin as such. Only a few investigators make any mention of this, yet as a rule the quantity of toxin formed is so small as to be of no practical value.

In both the older and the more recent literature there is a very large number of communications in which different "routine" culture media are described and attention is drawn to some of the important factors affecting toxin formation. The older literature is conveniently summarized in the monograph "Diphtheria" published by the Medical Research Council [1923]. After an extensive discussion of all the data then available the authors come to the conclusion: "At the moment it is only by adhering rigidly to certain formulae of technique that one may hope to arrive at a satisfactory product and, even with the most careful work and attention to detail, some batches of toxin do not come up to the standard of potency demanded by the maker of antitoxin. Certain factors are still beyond control, and until these are elucidated, the problem of making toxin on an entirely satisfactory basis will not be solved."

In spite of the very great amount of work which has been carried out on this subject since 1923, the verdict pronounced above still holds in its entirety.

A very extensive study on the formation of toxin was carried out by Pope [1932]. He started from a so-called "semi-synthetic" medium, in which Difco-proteose-peptone was employed as the source of nitrogen in conjunction with a few inorganic salts. Various sources of carbon such as sugars, organic acids etc., were added to a relatively simple "standard solution" and the effects of these additions on the formation of toxin investigated. Further attention must be drawn to a publication by Ström [1935]. This investigator examined the influence of various substances, when added to a simple stock solution, on the course of *p*H and the formation of toxin after inoculation with a diphtheria culture. The problem of the biochemistry of toxin production will be elucidated only when the following conditions are fulfilled.

1. The "stock solution" used must be as simple as possible; for this purpose the "semi-synthetic" medium described by Pope is eminently suitable.

2. An attempt must be made not only to obtain a qualitative, but as far as is possible, a quantitative idea of the processes involved.

3. The experiments must be carried out in direct connexion with toxin formation. Thus a medium of simple composition may be chosen, but a definite amount of toxin must be formed in this medium during incubation after inoculation with a diphtheria culture. Experiments in which no toxin is formed may of course be of value as supplementary evidence or as controls, but they are not essential.

In this communication we shall describe our experiments on the questions how rapidly sugars (glucose and maltose) are decomposed by diphtheria bacilli when added to a simple culture medium, and how this decomposition affects *p*H and the formation of toxin.

Method of experiment.

As culture medium use was made of the "semi-synthetic" medium of Pope, having the following composition:

Magnesium sulphate ($MgSO_4$, $7H_2O$)	0·2 g.
Calcium chloride ($CaCl_2$, $6H_2O$)	0·1 g.
Sodium phosphate (Na_2HPO_4, $2H_2O$)	1·0 g.
Potassium phosphate (K_2HPO_4)	1·0 g.
Glacial acetic acid	3·0 ml.
Difco proteose-peptone	20·0 g.
Distilled water	1000 ml.

All the constituents were dissolved without heating. *p*H was adjusted to 8 with 25 % NaOH solution and the sugar (glucose or maltose) added. The resulting solution was then filtered through paper and sterilized by filtration through a Seitz Entkeimungs-filter.

Culturing was carried out in large Fernbach flasks, having a diameter of 40 cm. and a height of 7 cm. Two litres of liquid culture medium were used in each flask. The liquid layer had a thickness of 2 cm. so that there was a very favourable ratio between surface and the thickness of the layer. The very wide necks of the flasks were closed by means of cotton-wool plugs carrying a tube about 12 mm. in diameter. After the desired quantity of sterile liquid had been introduced into the flask, this tube was closed by means of a rubber stopper through which a glass siphon tube passed. By means of this tube, fitted with a rubber tube, pinchcock, and glass protector, a sample could be taken at any desired time under sterile conditions. The sterility of the solution was checked by incubating the medium for two or three days before inoculating.

The strain used was the well-known Park-Williams 8, which is regularly used in this laboratory and which we obtained initially from Tomcsik. A day before the experiment, the strain was subcultured into fresh Löffler serum tubes and the 24-hour old culture suspended in salt solution and inoculated into the various flasks. Incubation took place at 35–36°.

Sampling was carried out as follows. First of all about 8 ml. of liquid were collected in a sterile measuring cylinder (10 ml. capacity) and, this being the volume of the siphon, it was thrown away. The necessary amount of liquid was then siphoned into a larger sterile measuring cylinder.

The following points may be mentioned as regards the methods of analysis. The glucose and maltose determinations were carried out by the method of Luff described in detail by Schoorl [1929; 1930], after the peptone had been precipitated with phosphotungstic acid [see Seibert, 1926; Tasman & Pot, 1934]. The 2 % peptone solution used showed a very small amount of reduction with the sugar test solution, which was included in each determination as a blank correction.

The pH was determined with a hydrogen electrode against a saturated calomel electrode, in an apparatus as used by Smit [1928].

The formation of toxin was examined by means of Ramon's [1923] flocculation test carried out at 50° and expressed as $\frac{1}{\mathrm{Lf}}$, determined against a serum dilution containing 100 antitoxin units per ml. By making use of 5 ml. quantities of toxin in each flocculation tube, a difference of 0·2 in the $\frac{1}{\mathrm{Lf}}$ value can be determined with certainty for values of $\frac{1}{\mathrm{Lf}}$ below 3.

All the experiments described in this paper were carried out at least in duplicate. Taken on the whole, the agreement between parallel experiments was very good. Only one experiment of each type is quoted.

In the first place, the above-mentioned values were determined in the Pope bouillon containing sodium acetate formed during the neutralization of the acetic acid. The results are given in Fig. 1.

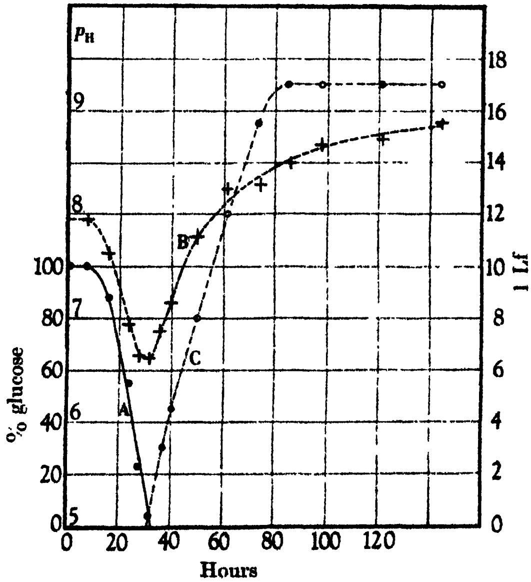

Fig. 1 (Exp. 1). Pope medium with acetate and 0·2% glucose. A, Glucose; B, pH; C, 1/Lf.

From these results it appears that the glucose disappears from the liquid in about 34 hours, whilst the formation of toxin begins before this time. The formation of toxin takes place at a practically constant rate until the maximum is reached, and after this time no further increase in the $\frac{1}{\mathrm{Lf}}$ value occurs. In all our experiments we have noted the same behaviour. In the early stages, the hydrogen ion concentration follows the decomposition of the sugar. As soon as the latter has disappeared, the well-known reversal occurs, the pH rises, rapidly at first and then more slowly. A possible explanation of this behaviour will be referred to later.

In order to study the effects of the acetate and the glucose present in the first experiment separately, two experiments were carried out in which either the acetate or the glucose was left out. The results of these experiments are collected together in Figs. 2 and 3.

A comparison of Figs. 2 and 3 with the graph in Fig. 1 shows that the decomposition of glucose in the second experiment, in spite of the absence of acetate, takes place with practically the same velocity as in the first experiment. In this case again, the formation of toxin commenced before the glucose had completely disappeared from the solution, but in Exp. 2, and to a more marked degree in Exp. 3, the amount formed was considerably less than in Exp. 1. The *p*H curve in Exp. 2 is similar in form to that in Exp. 1. In Exp. 3, the small decrease in *p*H at the beginning is probably due to the removal of traces of carbohydrates, which are present in the usual brands of peptone and which cause the formation of small amounts of acid by their decomposition.

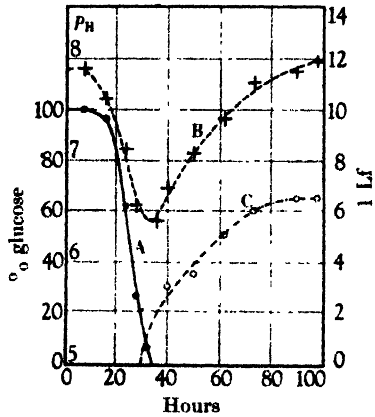

Fig. 2.

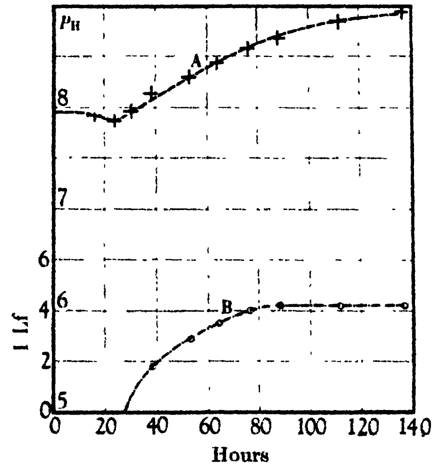

Fig. 3.

Fig. 2 (Exp. 2). Pope medium with 0·2% glucose, without acetate. A, Glucose; B, *p*H; C, 1/Lf.

Fig. 3 (Exp. 3). Pope medium with acetate, without glucose. A, *p*H; B, 1/Lf.

To complete this series Exp. 4 was carried out in which the additions of both acetate and glucose were omitted. The result is given in Fig. 4.

As regards the formation of toxin, this is again much less than in any of the previous experiments. With regard to the course of *p*H, especially the small decrease at the beginning of the experiment, the same remarks apply as in Exp. 3.

Since it appeared from various preliminary experiments which we carried out, in agreement with most of the data in the literature, that the optimum concentration of glucose was 0·2 %, it was of interest to try the effect of increasing the amount of glucose. Exp. 5 was therefore carried out with 0·5 % glucose (Fig. 5).

Although the diphtheria bacteria are able eventually to decompose this relatively large amount of glucose, the *p*H falls so much that, when all the glucose has disappeared from the solution, the conditions are too unfavourable for further development, so that there is no longer a question of *p*H reversal. Toxin is formed only in very small quantities and, as the high degree of acidity indicates, is destroyed as the experiment continues; at least it can no longer be determined by the flocculation test.

In recent years, the use of maltose instead of glucose in diphtheria culture media has been stressed more and more. In general, better results are obtained than was previously the case with glucose. Ström and others are of the opinion that the reason for this is that the maltose will be slowly converted into glucose, so that the diphtheria bacteria have ample opportunity for acting further on the decomposition products of glucose, whilst a smaller decrease in *p*H occurs. At the same time the growing culture is in contact with a small amount of glucose for a longer time.

Since preliminary experiments had indicated that the diphtheria bacteria could tolerate a concentration of 1 % of maltose in the Pope medium, Exps. 6 and 7 were carried out in which 1·0 % and 0·3 % respectively of maltose were added to the medium. This latter concentration was chosen in connexion with a routine medium to which a similar quantity of maltose is added (see Figs. 6 and 7).

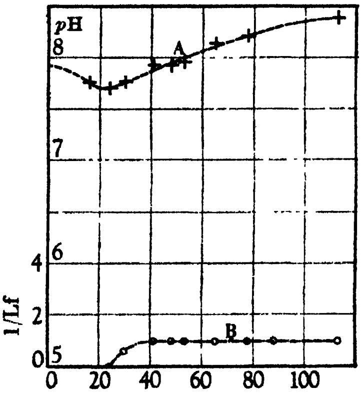

Fig. 4.

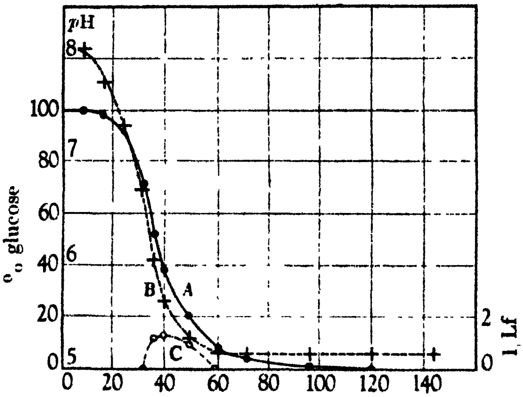

Fig. 5.

Fig. 4 (Exp. 4). Pope medium without acetate and without glucose. A, *p*H; B, 1/Lf.

Fig. 5 (Exp. 5). Pope medium with acetate and 0·5 % glucose. A, Glucose; B, *p*H; C, 1/Lf.

The maltose used was, with one exception (Exp. 6), supplied by the firm of Hoffmann La Roche & Co., Basel, and had the following characteristics: colour: white; solubility in water (1 : 1): complete; reaction to litmus: neutral; ash content: trace; $[\alpha]_D^{20^\circ}$ ($c = 5{\cdot}0$ % in water): 128·4° (lit. 128·5°). No glucose could be detected in a 10 % solution of this sugar by inoculation with a thick suspension of *Torula monosa* [Kluyver, 1914].

From a consideration of the figures, it follows directly that maltose is decomposed only very slowly and also that it never disappears completely from solution. The reason for the latter phenomenon must be left undecided for the moment. The *p*H decreases relatively little and then slowly rises. More remarkable in this connexion is the second rise in *p*H, which occurs after about 220 hours in Exp. 6. We have established that this is not fortuitous by various control experiments. If 1 % of maltose is added, the concentration falls almost linearly over a considerable period of time until, after about 170 hours, the fall becomes slower. The addition of maltose instead of glucose to the culture medium greatly favours the formation of toxin. This is most striking in Exp. 7 with 0·3 % of maltose. Further, it should be noted here, that the experiments with 0·3 % of maltose gave a more regular and consistent production of toxin than those with 1 % of this sugar.

Since the diphtheria bacterium is able on the one hand to decompose not more than 0·2 % of glucose without deteriment to the formation of toxin, yet on the other hand can decompose 1 % of maltose quite smoothly, the question

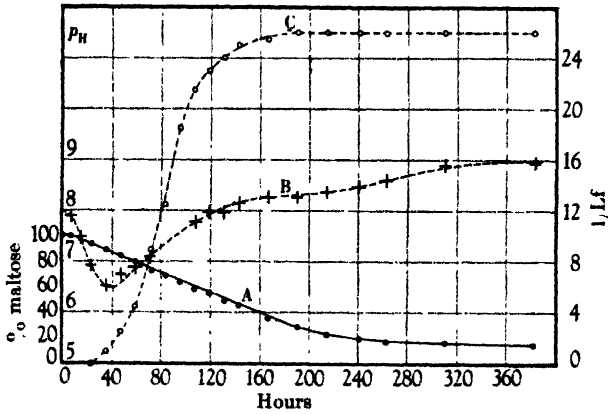

Fig. 6 (Exp. 6). Pope medium with acetate and 1 % maltose. A, maltose; B, *p*H; C, 1/Lf.

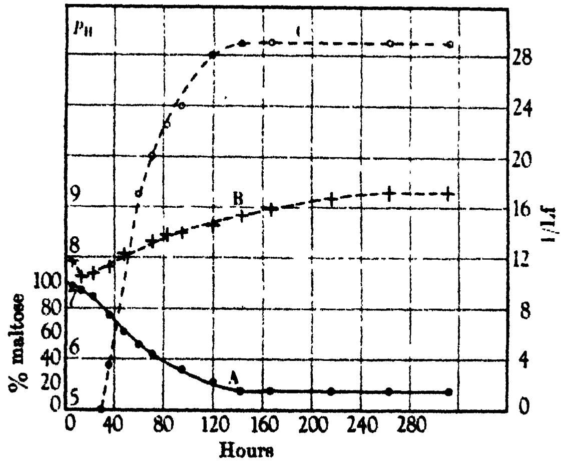

Fig. 7 (Exp. 7). Pope medium with acetate and 0·3 % maltose. A, maltose; B, *p*H; C, 1/Lf.

arises as to what the power of decomposing so much more maltose must be ascribed. Ström's opinion on this question has already been stated (p. 1760).

On the grounds of the ratio 0·2 % glucose : 1 % maltose, it may be supposed that the decomposition of glucose can proceed five times as rapidly as its formation from the mother substance, maltose. It appeared to be of interest to test this hypothesis experimentally by measuring the ratio of the two velocities more accurately. For this purpose Exp. 8 was carried out. To a stock solution containing acetate, 0·2 % of glucose was added to one half and 0·2 % of maltose to the other. Each solution was then divided between two flasks (each of two litres capacity). At definite times the sugar content was determined in all four flasks; in these experiments the pH and $\frac{1}{\text{Lf}}$ determinations were omitted. The results are given in Fig. 8, with regard to which it should be mentioned that the relative concentrations of maltose and glucose shown are the means of the separate values in the duplicate experiments.

The ratio of the velocities of the decomposition of glucose and the hydrolytic fission of maltose was now derived graphically as follows. The decomposition of glucose takes place during the interval of time between 15 and 35 hours after inoculation (i.e. the steepest part of the curve) with practically constant velocity. Thus this occurs between the points A and B. During this period, the relative concentration of glucose falls from 95% to 12%, i.e. by 83%. During the same interval of time, the concentration of maltose falls from 94% to 81%, i.e. by 13% between the points C and D. Thus roughly, the ratio of the velocities can be calculated as 83/13 = about 6. In other words, the amount of glucose formed by the hydrolytic fission of maltose is decomposed six times more rapidly than it is formed from maltose. This result agrees very well with the ratio figure ≧ 5 obtained above from very superficial considerations.

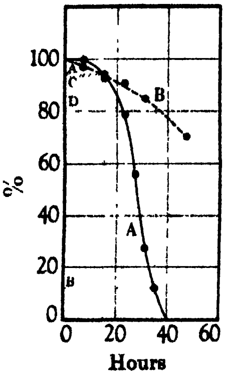

Fig. 8.

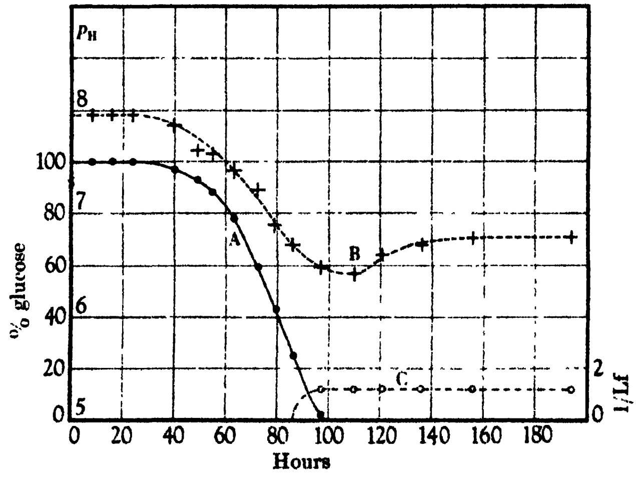

Fig. 9.

Fig. 8 (Exp. 8). Pope medium with acetate and 0·2% glucose, or 0·2% maltose. A, Glucose; B, maltose.

Fig. 9 (Exp. 9). Pope medium with acetate and 0·2% glucose. Proteose-peptone substituted by Witte peptone. A, Glucose; B, pH; C, 1/Lf.

On the grounds of these facts we should not expect to be able to detect glucose along with maltose during the incubation of substrates to which maltose has been added.

We repeated an experiment with the Pope medium containing 1% of maltose besides acetate. Samples of 500 ml. were taken after 9, 21 and 312 hours' incubation and each evaporated on the water-bath to ten times the initial concentration. The latter solutions contained 9·17, 8·60 and 1·76% of sugars, respectively, calculated as maltose. Dr L. H. C. Perquin very kindly tried under the supervision of Prof. A. J. Kluyver to determine biochemically any glucose present along with the maltose but was unable to do so. To both these gentlemen we wish to take this opportunity of expressing our heartiest thanks for their collaboration.

So far, these substrates have all contained the same source of nitrogen, namely, Difco-proteose-peptone. It was important to try other sources of nitrogen in this investigation. In the first place (Exp. 9, Fig. 9), we chose the well-known brand of Witte peptone of Rostock for this purpose. The media prepared for these experiments had exactly the same composition as the Pope medium, except that 2% of proteose-peptone was replaced by 2% of Witte peptone.

The poor growth and small film formation were striking. This brand of peptone, although fairly generally used previously for the preparation of diphtheria culture media, is very poorly suited for use as the sole source of nitrogen and stands in this respect very far behind Difco-proteose-peptone. Quantitatively this shows itself in a considerably reduced and very slow decomposition of sugar. Also the *p*H falls slowly and shows only a very feeble reversal. The formation of toxin is extremely small, but it is worth noting that, even in this experiment, the formation of toxin begins before the glucose has completely disappeared from the solution.

Finally two other culture media were examined in this investigation (Exps. 10 and 11, Tables I and II), which for convenience will be called by the names under which they are known in our Institute. The Tomcsik medium is prepared chiefly by the well-known recipe of Martin, meat extract + peptone from pig's stomach with the addition of 0·5% of sodium acetate and 0·2% of glucose, *p*H 7·6, whilst the Bandoeng medium differs from the one just described only in a few minor particulars, except that in place of 0·2% of glucose, 0·3% of maltose is added, *p*H 8. Since these results give graphs, which are almost identical with those in Figs. 1 and 7, the latter may be referred to in this case.

It appears that these experiments agree very closely with the corresponding experiments using the original Pope medium and 0·2% of glucose and 0·3% of maltose, respectively (see Figs. 1 and 7), and therefore call for no further comment.

A summarized version of the results is given in Table III.

DISCUSSION.

It appears that the diphtheria bacteria are able to decompose glucose completely at a maximum concentration of 0·5%, whilst maltose never disappears completely from the substrate. It is difficult to see the reason for this phenomenon. In spite of satisfactory growth at the beginning and during the first few days of the experiment, at a certain time a retardation or cessation of metabolism occurs, and the sugar still present is decomposed no further. Possibly factors connected with the nitrogen metabolism play a part here. However, until more data on this subject are forthcoming, there is little more to say.

As regards the velocity of decomposition of glucose, this appears to be practically independent of whether or not acetate is present, but depends far more on the nature of the source of nitrogen present. In this connexion, a combination of meat extract + pig's stomach peptone as used in the Tomcsik medium, is practically of equal value to the Difco-proteose-peptone used in the Pope medium. Witte peptone, however, which gives a very poor growth and so brings about a very slow decomposition of the sugar, behaves quite oppositely. The fact that the sugar in a Pope medium with 0·5% of glucose is decomposed rapidly at first but later is completely used up only slowly, is probably due entirely to the high degree of acidity developed in the later stages of the experiment.

Considering now the alteration in the hydrogen ion concentration in the various experiments, the following statement may be made. The *p*H falls more or less considerably in all cases where glucose is decomposed by the bacteria either

Table I. *Tomcsik medium with 0·5 % sodium acetate and 0·2 % glucose.*

		Glucose		
Hours of cultivation	pH	Absolute quantities in %	% of initial quantities	$\frac{1}{Lf}$
0	7·46	0·199	100	—
8	7·46	0·199	100	—
16	7·40	0·199	100	—
24	7·18	0·179	90	—
28	6·95	0·164	82	—
32	6·57	0·141	71	—
36	6·23	0·100	50	—
40	5·88	0·057	29	1·0
50	6·55	—	—	6·5
62	7·61	—	—	10
73	8·25	—	—	13
86	8·35	—	—	13
97	8·47	—	—	13
121	8·76	—	—	13
145	8·92	—	—	13

Table II. *Bandoeng medium with 0·5 % sodium acetate and 0·3 % maltose.*

		Maltose		
Hours of cultivation	pH	Absolute quantities in %	% of initial quantities	$\frac{1}{Lf}$
0	7·94	0·276	100	—
12	—	0·276	100	—
24	7·88	0·276	100	—
48	7·63	0·261	95	—
60	7·56	0·229	83	1·0
72	7·85	0·190	68	—
74	—	—	—	7·8
79	7·99	0·174	63	9
84	8·04	0·169	61	—
96	8·14	0·145	53	—
120	8·49	—	—	—
123	—	—	—	18
145	8·64	0·112	41	19
169	8·87	0·105	38	19
216	9·02	0·101	33	19
266	9·16	0·101	33	19

Table III. *Summary of velocities of sugar breakdown in the various culture media.*

Medium	Specimen of peptone	Presence of acetate	Sugar used	Time at which the sugar has disappeared hours
Pope	Difco-proteose	+	0·2 % glucose	32
	,,	−	0·2 % glucose	34
	,,	+	0·5 % glucose	120
	,,	+	1 % maltose	∞
	,,	+	0·3 % maltose	∞
	Witte	+	0·2 % glucose	98
Tomcsik	Pig's-stomach	+	0·2 % glucose	43
Bandoeng	Pig's stomach	+	0·3 % maltose	∞

directly or after formation from maltose, doubtless owing to the production of various acids by the degradation of the glucose. The fall in *p*H is greater, the greater the amount of glucose available for decomposition. In experiments using 0·2 % of glucose, the lowest *p*H observed was 5·88 in the case of the Tomcsik medium, whilst the experiment with 0·5 % of glucose showed a fall to 5·15. It is of course obvious that this lowest *p*H value and the course of the *p*H curve are dependent on the buffer capacity of the medium employed (the kind of peptone used, the presence or absence of acetate etc.).

What causes this rise in *p*H, which always occurs in cases that are physiologically normal for the bacteria in question? The compilers of the monograph "Diphtheria", already mentioned, give it as their opinion on this point, that after the acids have reached a certain concentration, the bacteria oxidize them to CO_2 (and H_2O), which combines with the alkali originally present to form carbonate and bicarbonate and so explains the inflexion of the *p*H curve. Although an experimental proof of this hypothesis is still wanting, a fact which strongly supports it is, that in all cases of direct decomposition of glucose, the minimum *p*H coincides for all practical purposes with the disappearance of the sugar from the medium. One must suppose therefore that glucose, as such, is an easily available source of energy of great value, which is first assimilated practically completely, before the less readily accessible, poorer source of energy, the acids, is consumed. The facts that the *p*H in the experiments with maltose decreased less and that the minimum in the *p*H curve was less well defined, is also in agreement with the above. In these cases of indirect glucose decomposition, both processes occur simultaneously, splitting of glucose into acids and oxidation of the latter to carbonates. Finally, in this connexion, an attempt must be made to find an explanation for the second rise in the *p*H curve observed in experiments with 1 % of maltose. In this case also, the explanation must be considered hypothetical. When this second rise occurs (after about 220 hours, see Fig. 6), the decomposition of sugar and the formation of toxin are practically at a standstill. Thus, at this time, presumably, there will be no more acid formed in the solution, which may cause a rise in *p*H by subsequent oxidation to carbonate. The idea suggests itself that this second rise in *p*H might be the result of the formation of extra ammonia from the peptone present.

The formation of toxin can be considered under three headings: (*a*) the time at which toxin formation begins, (*b*) the velocity with which formation takes place, (*c*) the maximum value reached.

As regards the first point, experiments with glucose give the simplest picture. In all these experiments toxin formation occurs at the time when the glucose practically disappears from the solution. The time at which toxin formation begins (which can only be approximately determined by graphical methods) is given in Table IV, along with the corresponding maximum values.

The figures indicate clearly that this setting off point, both in experiments with maltose and glucose, coincides with the minimum *p*H.

The question now arises whether anything can be deduced from these phenomena regarding the nature of the toxin formation process. Without going into further details, as experimental data on this point are still wanting, we should like to consider the formation of toxin chiefly as a result of dissimilatory nitrogen metabolism, which begins at the moment when the carbon sources are mostly used up.

In those cases where toxin is formed in reasonable amounts, the velocity of the process is almost constant until the maximum is reached, when it suddenly

Table IV. *Summary of toxin production in the various media.*

Medium	Beginning of toxin-production after hours	Maximum toxin-value
Pope, proteose-peptone, acetate, 0·2 % glucose	30	17
Pope, proteose-peptone, no acetate, 0·2 % glucose	28	6·5
Pope, proteose-peptone, acetate, no glucose	30	4·2
Pope, proteose-peptone, no acetate, no glucose	24	1·0
Pope, proteose-peptone, acetate, 0·5 % glucose	32	1·3
Pope, proteose-peptone, acetate, 1 % maltose	24	26
Pope, proteose-peptone, acetate, 0·3 % maltose	31	29
Pope, Witte peptone, acetate, 0·2 % glucose	85	1·2
Tomcsik, pig's stomach, acetate, 0·2 % glucose	49	13
Bandoeng, pig's stomach, acetate, 0·3 % maltose	58	19

falls to zero. The cause of this more or less abrupt cessation in the production of toxin is difficult to diagnose.

This phenomenon may find its origin in the utilization of a necessary constituent for toxin formation, or the attainment of too high a concentration of one or other of the products of metabolism. The absolute value of the velocity (measured by the steepness of the toxin curve) is of course dependent on various factors connected with the metabolism.

As shown by Table IV, the composition of the medium plays a very important part in determining the maximum value obtained in the various experiments for the concentration of toxin. The pronounced effect of the absence or presence of acetate in the medium shows that the decomposition of definite carbon compounds is of great importance for the formation of toxin.

SUMMARY.

1. A series of experiments was carried out with *C. diphtheriae* to determine the velocity of decomposition of the sugar, the changes in the hydrogen ion concentration and the formation of toxin, glucose and maltose being used as sugars, whilst various brands of peptone were employed as sources of nitrogen.

2. The diphtheria bacteria are able to assimilate glucose up to a concentration of 0·2 % without detriment to the formation of toxin. If more glucose is added (0·5 %), the total amount of sugar is decomposed, but such a large amount of acid is formed that the production of toxin is almost completely inhibited. The decomposition is slower than with the smaller concentration of sugar. On the other hand maltose is tolerated in much higher concentrations. The cause of this phenomenon appears to lie in the fact, that the velocity of decomposition of glucose by diphtheria bacteria is about six times greater than the velocity of formation of glucose by the hydrolysis of maltose.

3. In the decomposition of the sugar, the *p*H first of all falls until a point is reached when the sugar (glucose) disappears from the solution, and then it rises again, owing probably to oxidation of the acids, formed initially from the glucose, to CO_2 and H_2O, which combines with the alkali originally present to basically reacting carbonate and bicarbonate.

4. The formation of the toxin proper begins at the moment when the sugar (glucose) practically disappears from the solution, continuing during a definite period with practically constant velocity, to cease quite suddenly. The $\frac{1}{Lf}$ value, measured by means of Ramon's flocculation test, does not decrease on continued incubation of the culture.

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CCXLVII. SO-CALLED VIRTUAL ADRENALINE OF THE SUPRARENAL CORTEX.

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THE close structural relationship which adrenaline bears to the two naturally occurring amino-acids, tyrosine and phenylalanine, has focussed attention on both of these substances as possible biological precursors. The claim of Halle [1906] that adrenaline is formed when suprarenal tissue is incubated with tyrosine was shown conclusively by Ewins and Laidlaw [1910] however to be based on unsound evidence. Similar negative results have also been reported by Nikolajeff [1924] on perfusion of tyrosine through the surviving suprarenal gland: by Trendelenburg [1929] who treated the pulped gland with dihydroxyphenylethylmethylamine and also by Funk [1912] in similar experiments with 3:4-dihydroxyphenylalanine (dopa). Heard & Raper [1933] have shown that both dopa and N-methyldopa are stabilized against subsequent oxidation when perfused through the surviving adrenal gland whereas when exposed to the action of tyrosinase they yield small amounts of pressor bases (probably aminoacetocatechol and adrenalone respectively). Apart from the recent experiments of Schuler & Wiedermann [1935] which have not yet received confirmation there is then little satisfactory evidence that the synthesis of adrenaline actually takes place in the gland or that the latter contains any demonstrable precursor. It must be realized however that failure to demonstrate any such formation by minced or surviving tissue does not preclude the possibility of some change, necessary to the process as a whole, having taken place.

Another possibly significant factor is the distribution of adrenaline as between the cortex and medulla of the gland. The small fraction generally found in cortical extracts may be due in part to post mortem diffusion from the medulla and to the impossibility of effecting an absolutely "clean" separation of each type of tissue by dissection. For this reason and also because the tissues are morphologically independent little attention other than that of Abelous *et al.* [1905] has been paid to the possible role of the cortex in the elaboration of adrenaline. More recently Abelous & Argaud [1931, 1932, 1934] have concluded that adrenaline is merely stored in the medulla after it has been formed in the cortex from a parent substance "virtual adrenaline".

The purpose of the present communication is to describe attempts to reproduce these latter experiments and to study the effect of "virtual" adrenaline on the assay of suprarenal extracts.

EXPERIMENTAL.

Estimation of adrenaline.

The principal methods of estimating adrenaline are based either on (*a*) its oxidation to a "characteristic" red pigment, or (*b*) its reduction of Folin's phosphotungstate reagent.

Both of these methods are likely to give erroneously high results in the assay of suprarenal extracts if other chemically allied substances are present. Heard [1932] has tested both types of estimation with the related compounds dopa, methyldopa and epinine using the Folin method [Folin *et al.*, 1912] and the various oxidizing agents proposed by Vulpian [1856], Comessatti [1908] and Ewins [1910]. In each case the tint and intensity of colour were identical with those given by an equimolecular concentration of adrenaline. Folin's method is liable to further error since the reagent is reduced by uric acid and other extractives including, particularly, ascorbic acid which is present in both the medulla and cortex of the gland. In the present experiments the persulphate method of Ewins as modified by Barker *et al.* [1932] has been used since a careful comparison made by them showed that this gave results most consistent with the physiological assay (pressor activity).

In order to gain additional information as to the mechanism of adrenaline formation the total amount of catechol substances also present at different stages of the process was estimated by the ammonium molybdate method of Rae [1930]. In deproteinized cortical extracts colour matching against a standard adrenaline solution is made difficult by the presence of ascorbic acid which, with molybdate, gives a similar yellow colour turning finally an intense blue after a few minutes. The error on this account is not serious provided that the solution is assayed immediately after adding the reagent. In a test experiment, minced cortical tissue of the bullock, after being shaken in air for 3 hours with 0·5% sodium carbonate, was found by indophenol titration [Birch *et al.*, 1933] to contain only 0·4 mg. ascorbic acid/g. tissue. The effect of this concentration could be ignored since the standard solution, equivalent to four times this concentration, did not itself give an effective colour with molybdate.

The hypothesis of "virtual" adrenaline.

The results of Abelous & Argaud [1931, 1932, 1934] already referred to may be summarized as follows:

(*a*) If the minced cortical tissue of horse suprarenal gland is shaken at room temperature for several hours in 3% Na_2CO_3 then both chemical and physiological assays show an increase in adrenaline content. Under the same conditions all the adrenaline in medullary tissue or in a mixture of medullary and cortical tissues is destroyed. These effects are not dependent on the presence of tissue and take place in deproteinized acid extracts subsequently made alkaline.

(*b*) A deproteinized *aqueous* extract of cortex contains much more adrenaline measured physiologically (pressor effect) than by colorimetric assay; whereas in an aerated alkaline extract the pressor response is the same as before whilst the chemical assay shows a marked increase.

Hence it is concluded that the suprarenal cortex contains very little free adrenaline but a relatively large quantity of "virtual" adrenaline which has a similar pressor activity, does not respond to colorimetric tests and is converted into adrenaline by oxidation in alkali. In the medulla the proportions of free and "virtual" adrenaline are reversed.

The first two attempts to repeat these experiments were made with the suprarenal glands of the bullock. These were de-fatted and dissected immediately on arrival in ice from a local abattoir. As noted by Schild [1933], numerous assays made in the course of this investigation indicate that elaborate precautions against the decomposition of active material are unnecessary. The tissue was minced and thoroughly mixed and samples of 10 g. medulla and 20 g.

cortex weighed into wide-necked 200 ml. bottles and each pair treated separately as follows, except in IV and V which refer to *mixtures* of 10 g. medulla and 20 g. cortex:

I. (Controls). Extracted with $N/10$ H_2SO_4.
II and IV. Shaken for 1½ hours in air in 0·1 % Na_2CO_3.
III and V. Shaken for 4 hours in air in 0·1 % Na_2CO_3.

At the end of the process each sample was acidified with 10 ml. N H_2SO_4 to stabilize the adrenaline then corked and kept overnight at 0°. The mixture was filtered through muslin and the residue ground with sand and re-extracted. The total crude filtrate was heated nearly to boiling and the protein removed by adjusting the pH to 5·5 (methyl red). A water-clear, almost colourless extract was thus obtained from which aliquots were removed for the estimation of adrenaline and of total catechol substances. At the same time the adrenaline content was assayed physiologically by estimating the pressor activities of the deproteinized solutions by intravenous injection into a pithed cat. The results are presented in Table I, the material responding to each test being reckoned as mg. adrenaline in the whole sample of tissue.

Table I.

Sample		Persulphate	Molybdate	Pressor assay
I.	M	41	144	185
	C	10	21	15
II.	M	43	136	157
	C	10	18	15
III.	M	41	126	149
	C	8	18	12
IV.	(M+C)	28	146	202
V.	(M+C)	26	150	185

In each Table, unless otherwise stated, M = Medulla (10 g.); C = Cortex (20 g.).

As there was doubt at this stage as to the validity of the persulphate estimation the marked discrepancy shown between the chemical and physiological assays will be discussed later. If attention is confined to the latter it will readily be seen that they in no way bear out the results of Abelous and Argaud previously discussed. If MI and CI (*acid* extracts)[1] be accepted as controls for the remaining samples (*alkaline* extracts) then it appears that the adrenaline present in the medulla does not wholly disappear, but rather is the stabilizing effect of the system emphasized. Thus after 1·5 hours' exposure to air in alkaline solution, 85 % of the pressor activity still remained and only a further 5 % was lost at the end of 4 hours. Conversely the activity of the cortical tissue does not show a marked rise but a fall of about 20 % as in the medulla. In the samples of mixed tissue the predominant effect was the same.

As the conditions had been made less drastic so as to minimize the destructive effect of oxidation, the experiment was repeated adhering more closely to the conditions originally laid down. The treatment was therefore carried out in 3 % Na_2CO_3, to which a little octyl alcohol was added, by drawing a steady stream of air through the bottles which were immersed in a thermostat at 34–35°. The control extracts were made under hydrogen. The effect was also studied on protein-free acid extracts which were assayed before and after alkaline aeration thus eliminating errors due to lack of homogeneity in sampling the tissue. The treatment is summarized below and the results presented in Table II.

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[1] The difference between acid and alkaline extracts is probably not significant. Cf. Table II.

I. (Controls.) Extracted with alkali under hydrogen for 4 hours.
II. Aerated in alkali for 4 hours.
III. Extracted with $N/100$ H_2SO_4 under hydrogen.
IV. Extracted with water under hydrogen.
A. Deproteinized aliquot of IIIC made alkaline and aerated.
B. As in A with addition of 10 g. fresh medulla before aeration.

Table II.

Sample		Persulphate	Molybdate	Pressor assay
I.	M	50	126	152
	C	10	19	13
II.	M	52	136	130
	C	6	20	7
	M + C	52	182	124
III.	M	14	125	104
	C	7	17	13
IV.	M	39	119	118
	C	10	11	9
	A*	0	0	0
	B*	—	—	40

* These analyses are referred to the whole of IIIC, as "aliquots". Chemical analysis of B was impossible on account of the deep colour of the solution. As further decomposition appears to have taken place before the physiological assay could be made the value obtained represents only a minimum figure.

The pressor assays of samples I and II indicate substantially the same results as before save that the failure of the cortical tissue to general adrenaline is even further emphasized since after 4 hours' treatment the destruction in the cortex (44 %) was three times as great as in the medulla and nearly twice as much as in a mixture of both. The analyses A and B and those under III form a parallel experiment conducted on a deproteinized solution and show the same effect. Finally reference to the analyses under II and IV shows that in an aqueous extract of cortex both chemical assays are as high as the physiological assay while the only apparent effect of aeration has been to oxidize the adrenaline side chain without destroying the catechol nucleus so that the molybdate assays IC and IIC are the same while the pressor assay of IIC is notably lower than IC. The results therefore lend no support to the hypothesis of "virtual" adrenaline.

Consideration of the chemical estimations shows that the persulphate figures vary too widely from the pressor assays to allow any useful analysis. On the other hand the molybdate estimations which in the cortical extracts are always in excess of the pressor assay show in the medullary extracts a mean difference of only 7 %. This suggests the presence in the cortex of a catechol substance other than adrenaline. The discrepancies between physiological and colorimetric assays of suprarenal extracts noted by numerous investigators including, more recently, Frowein [1922] and Maiweg [1922], support this belief. Annau *et al.* [1932] postulated the existence in the medulla of an unstable derivative "novadrenine", having many times the activity of free adrenaline. Euler however [1933], using a Pulfrich "Stufenphotometer" and a modified form of the Vulpian (iodine) reaction, concluded that in medullary extracts there is no significant difference between chemical and physiological assays. Less perfect agreement was reported by Schild [1933] in a comparison of the iodine, persulphate (modification of Barker *et al.*) and Folin methods as applied to medullary extracts. In each case, the physiological exceeded the colorimetric assay and where comparison of the latter was possible best agreement was obtained in the order named. Schild has criticized the persulphate method adversely because of the somewhat haphazard development of colour which, even in pure adrenaline solutions, makes direct comparison impossible, although reasonable results can be obtained by taking the maximum "red" reading in a tintometer. The analyses reported in Tables I and II were carried out by matching in a colorimeter against a standard solution made by weighing out pure adrenaline and dissolving it in slightly more than 1 equiv. of dilute H_2SO_4. A standard time at room temperature of 2½ hours was found to be sufficient for development of the colour which is stable for many hours in the absence of air.

Examination of various inhibitory factors which might operate suggested either incomplete removal of protein by the method adopted or variable temperature effects. An extract of whole gland was therefore made with dilute H_2SO_4, and the precipitation of protein completed at *p*H 5·5 by adding a little trichloroacetic acid to the hot solution. Persulphate estimation at various temperatures up to 40° was in each case wholly unsatisfactory as compared with the iodine method of Schild [1933] who obtained similar results for both methods of deproteinization. The only effect of elevated temperature was to strengthen the yellow component of the colour. The persulphate method therefore cannot be recommended except when used with a tinotometer, nor can the analyses by this method reported in Exps. 1 and 2 be regarded as satisfactory.

It was thought that a final commentary both on the value of this method and on the existence of "virtual" adrenaline should be made by carrying out a more strict comparison using the adrenal glands of the horse. Quantities of 11 g. minced cortex and 4 g. medulla were therefore treated as follows for 3 hours:

I. Extracted with $N/20$ H_2SO_4.
II. Extracted with H_2O.
III. Aerated in 1 % Na_2CO_3.
IV. Aliquot of cortical extract from I aerated in alkali.

The results are given in Table III (mg./10 g. tissue).

Table III.

Sample	Persulphate	Iodine		Molybdate	Pressor assay
I. C	2·7	4·9	5·2	—	6·8
II. M	32·5	85	85	93	95
C	5·5	4·2	4·4	—	4·9
III. M	0	0	0	—	10·3
C	2·6	—	3·0	—	3·6
IV. C*	—	—	0	—	0·8

* Referred to the whole of CI as "aliquot".

In these solutions 6 hours at 22–24° were required for maximum colour development by persulphate. The estimations by iodine were duplicated so that the effect of varying certain conditions such as reaction time, excess of reagent and adrenaline concentration could be gauged. The high ascorbic acid content made application of the molybdate reaction to most of the solutions impossible. The analyses, presented in Table III, bear out the previous failure to reproduce the experiments on which the concept of "virtual" adrenaline is founded, for which at present no satisfactory explanation can be advanced.

Distribution of catechol substances in the suprarenal glands of the bullock.

As the discrepancy between physiological and chemical estimations of adrenal extracts is generally attributed to the latter it must be stated that the iodine method gives readily reproducible results when carried out under the conditions stated by Schild and that the colour developed is constant over wide variations in the amount of reagent added. The figures presented so far suggest that one source of error not previously examined may lie in the distribution in the gland of catechol substances other than adrenaline, i.e. in the difference between the iodine and molybdate assays as shown in Table IV. The first extract of each pair was made with $N/20$ H_2SO_4 and the second with 5% trichloroacetic acid.

Table IV. *Comparison of assays for medulla and cortex (mg./g. tissue).*

	Adrenaline	Total "catechol"	Pressor assay
Medulla	11·0	12·6	—
	11·3	13·3	12·1
Cortex	0·77	1·24	—
	0·71	1·15	0·65

The cortex apparently contains about 40% of its catechol material in a form which has little or no pressor activity. The proportion is much less in the medulla but is too great to be accounted for by possible admixture with cortical tissue in dissection. The following factors which might influence the molybdate estimation were further investigated:

Effect of pH. 4 ml. of medulla extract suitably diluted at *p*H 5·5 were treated with 5 ml. acetate buffer and 4 ml. reagent. The mean variation in colour intensity was shown to be only about 2% over the *p*H range 3·6–5·6 if the standard be developed at the same value.

In the cortical extract the effect seemed to lie in the opposite direction, owing possibly to the much greater interference of ascorbic acid which can be minimized by selecting a fairly high *p*H but one at which adrenaline itself is quite stable—say 5·4.

Effect of ascorbic acid. A test made as quickly as possible before further change showed that the yellow colour given by ascorbic acid is about one fortieth as effective as that of an equal weight of adrenaline and in fresh extracts would lead to a maximum error of about 6% in the cortex and 0·3% in the medulla. Other more sensitive reagents, such as Folin's, which are reduced by ascorbic acid give rise to more serious error. The quantitative separation of adrenaline and ascorbic acid cannot readily be carried out for routine analysis. Lead acetate has been used to remove the vitamin but there is disagreement as to the effective *p*H [Zilva, 1927; Smith & King, 1931; Emmerie, 1934]. 100 ml. cortex extract containing 50 mg. ascorbic acid were therefore treated with 20 ml. 10% lead acetate and adjusted with ammonia to *p*H 6·0, 7·2 and above 10·0. The procedure was unsatisfactory as each of the three precipitates was found to contain both "catechol" and ascorbic acid. A simpler method is to destroy the latter by aeration. According to Ahmad [1935], with ordinary exposure of such extracts oxidation commences about 3 hours after preparation. The effect on solutions kept sealed at 0° is shown in Table V (Exp. 1) by the increase in titre against a constant volume of indophenol solution.

Exp. 2 gives the analyses made 3 weeks after extraction of samples from another batch of glands. The yields are less but lie within normal variation

Table V. *Rate of oxidation of ascorbic acid at 0°.*

Exp.	Time in days	Titre (ml.)		M (mg./g.)		C mg./g.	
		M	C	Adrenaline	"Catechol"	Adrenaline	"Catechol"
I	0	4·3	2·3	—	—	—	—
	1·5	5·3	4·0	11·8	11·8	0·66	—
	6·5	14·3	113	—	—	—	1·08
II	—	—	—	9·1	10·2	0·41	0·74
	—	—	—	9·4	10·2	0·41	0·84
	—	—	—	8·9	9·9	0·42	0·82

[Schild, 1933]. In both groups the ratio of total "catechol" to adrenaline remains almost constant at 1·1 (medulla) and 1·7 (cortex) respectively. This suggests that the bio-synthesis of adrenaline may be regarded as a two-stage process in which an intermediate catechol substance is first elaborated by the cortex and is then carried into the medulla and converted into adrenaline. Further work along these lines is being carried out.

SUMMARY.

1. Oxidations have been carried out in alkaline media with the cortical and medullary tissues of the suprarenal glands of the horse and bullock. Chemical and physiological assays of the resulting solutions do not support the hypothesis of the existence in the adrenal gland of "virtual" adrenaline.

2. The distribution of catechol substances in the gland suggests that the cortex may play some part in the elaboration of adrenaline.

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CCXLVIII. NEW ZEALAND FISH OILS.
I. THE COMPOSITION OF EEL OIL (*ANGUILLA AUCKLANDII*).

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It has been suggested by Lovern [1932 *et seq.*] that the fats of freshwater fish are, within comparatively small limits, characteristic for that class and quite distinct as regards the relative proportions of component fatty acids from marine fats. So far as the authors are aware the data on this subject have been derived almost entirely from species inhabiting the Northern Hemisphere. The present work, however, suggests that this important generalisation with regard to the composition of freshwater fats can be extended to include species inhabiting the Southern Hemisphere.

In most parts of New Zealand, eels are the predominant freshwater fish. The two species known respectively as the short-finned or northern eel (*Anguilla australis*) and the long-finned or southern eel (*Anguilla aucklandii*) are noted for their large size when mature; specimens weighing 35 lb. or more being occasionally caught.

The composition of New Zealand eel oil does not seem to have been previously determined. Wiehr [1934] reports that the fat from *A. vulgaris* yielded 86·5 % fatty acids of which the solid acids separated by Farnsteiner's method comprised 20·3 %, whilst Ono [1935] gives qualitative data for the composition of Japanese eel oil. The general characteristics of various eel oils determined by previous investigators are given in Table I.

Table I. *The general characteristics of some eel oils.*

Species	Sap. equiv.	I.V.	Unsaponifiable (%)	Observer
A. rostrata	293·6	117·4	—	White [1912]
A. vulgaris	273·4	90·6–105·8	0·74	Wiehr [1934]
New Zealand eel	289·2	107·0	1·0	Denz and Shorland [1934]
Japanese eel (Spring)	284·7	146·2	—	Ono [1935]
„ (Summer)	290·7	158·9	—	„

Eel oil has been stated to contain not only vitamin D but also nearly as much vitamin A as good cod liver oil [Medical Research Council 1926–7]. In the case of New Zealand eels we have measured vitamin A blue values varying from 4·3 to 12·0. Most of the fat is contained in the body, the small amount of oil in the liver, however, appears to be extremely rich in vitamin A. A sample weighing 254 g. and comprising 19 livers, kindly placed at our disposal by Dr E. Marsden of the Department of Scientific and Industrial Research, Wellington, New Zealand, yielded 2 % oil of blue value 1300. For a sample of small eels (average weight 1 lb.) it was found that the livers comprised only 1·8 % of the total weight.

The sample used in this investigation was a specimen of *A. aucklandii* taken near Upper Hutt in the Wellington District of the North Island during February 1935. The following measurements may be given. Length, 4 ft. 3 in.; weight, 20 lb.; weight of liver 4 oz.;

$$\text{distances between dorsal fin and vent} \times \frac{100}{\text{length of fish}} = 8\,\%.^{1}$$

After removal of viscera, the bulk of which comprised liver, the remainder was steam-cooked, pressed and then extracted with light petroleum (B.P. 60–80°). The extract (7 %) freed from the last traces of solvent by steam distillation, gave the following general characteristics. Sap. equiv., 290·4; I.V., 122·5; % unsaponifiable matter, 0·80. In Table II, the data for the component fatty acids of eel oil determined according to the procedure of Guha *et al.* [1930] are compared with the summarised data for five freshwater fats analysed by Lovern [1932].

Table II. *Component fatty acids of freshwater fish oils expressed as molar percentages.*

	Saturated			Unsaturated				
	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
A. aucklandii	2·4	15·9	0·8	—	21·2 (−2·0 H)	46·3 (−2·6 H)	12·9 (−6·2 H)	0·5 (−? H)
Freshwater species (Lovern)	(3·4–5·6)	(13·4–16·0)	(tr.–1·9)	(0·6–1·8) (−2·0 H)	(19·0–22·4) (−2·0 H)	(37·4–44·5) (−2·8 to −3·4 H)	(12·2–13·8) (−6·7 to −7·5 H)	(0·0–6·0) (−7·5 to −9·2 H)

It will be observed that apart from minor differences such as the relatively low molar percentages of myristic acid and the high molar percentages of C_{18} unsaturated acids together with the low mean unsaturation of the C_{18} and C_{20} groups there is a general agreement between the composition of the eel oil examined and fats from typical freshwater fish in the Northern Hemisphere.

Until quite recently it appears to have been widely assumed that the C_{18} unsaturated acids of fish oils consisted of oleic acid together with linoleic and linolenic acids characteristic of plant and animal fats. Various investigators have, however, reported occasionally the occurrence of a tetraethenoid C_{18} acid in fish oils [cf. Toyama & Tsuchiya, 1929]. The only quantitative data with regard to the relative amounts of C_{18} polyethenoid acids in aquatic oils appear to be those of Green & Hilditch [1936]. These investigators have shown that cod liver and whale oils contain not more than traces of linoleic and linolenic acids, the polyethenoid unsaturation being due in part at least to octadecatetraenoic (stearidonic acid). On the other hand, the C_{18} acids of the grass-feeding carp were found to contain smaller proportions of stearidonic acid but definite amounts of linoleic and linolenic acids. In view of these results it seemed desirable to obtain further information with regard to the C_{18} unsaturated acids of eel oil. A purified C_{18} fraction (Sap. equiv. 308·8; I.V. 115·4) was prepared by refractionation of a C_{18} concentrate obtained during the distillation of the ethyl esters of the "liquid" acids. The corresponding acids (3·59 g.) were brominated and fractionated into ether-insoluble and petroleum-insoluble bromides as described by Green and Hilditch [1936]. The data thus obtained are summarised in Table III.

The C_{18} unsaturated acids (7·30 g.) were further examined by means of oxidation with alkaline permanganate (Hazura). The water-insoluble fraction (2·96 g.) M.P. 120–122° on recrystallisation from ethyl acetate gave dihydroxy-

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[1] This formula is used to distinguish between the two species; in the case of *A. australis* the value seldom exceeds 4 %.

Table III. *The bromo-additive products of C_{18} unsaturated acids of eel oil.*

	Weight	M.P.	Br %	Total acids %	I.V. (calculated from Br (%))
Insoluble in ether	0·52	>190° (d)*	67·0	4·8	322·1
Insoluble in petroleum	0·26	145–147°	61·4	2·8	252·3

* (d) with decomposition and charring.

stearic acid M.P. 129–130°. No tetrahydroxystearic acid was found in the water-soluble fraction. The high melting-point and bromine content of the ether-insoluble bromides point to the presence of stearidonic acid. In the case of the petroleum-insoluble bromides, the bromine content (61·4 %) is somewhat lower than required for hexabromostearic acid (63·3 %). However, in view of the failure to obtain tetrahydroxystearic acid and the low melting point observed it is quite possible that the petroleum-insoluble bromides represent incompletely brominated stearidonic acids. The composition of the C_{18} polyethenoid acids of eel oil thus appears to resemble that of cod-liver or whale oil.

SUMMARY.

The fat from a specimen of New Zealand eel (*A. aucklandii*) has been examined. The composition is shown to resemble closely that of the typical fresh-water fish examined by Lovern [1932]. Oxidation with alkaline permanganate and bromination respectively have shown the C_{18} unsaturated acids to contain in addition to oleic acid some stearidonic acid. No evidence, however, for the presence of either linoleic or linolenic acid has been found.

The authors wish to thank Mr B. C. Aston of this laboratory for encouragement and facilities. Thanks are also due to Prof. T. P. Hilditch of Liverpool University for his kind interest and invaluable assistance in connection with the calculation of percentages of the component fatty acids.

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CCXLIX. THE PROTEOLYTIC ENZYMES OF SPROUTED WHEAT. II.

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In a previous communication the author [1936] has described some of the principal characteristics of a proteinase and a dipeptidase which appear in the aqueous extracts of germinated wheat seeds. The aim of the present paper is to supply further data with regard to these two enzymes and to amplify certain of the findings already recorded, principally those concerned with the relative stabilities of the proteinase and dipeptidase and with the effect of cyanide upon enzymic activity.

Experimental.

The technique adopted was substantially the same as that already outlined, the Sørensen method of formaldehyde titration in two stages, (*a*) and (*b*), forming the basis of the investigation. In a few cases $N/10$ NaOH was employed in place of $N/20$ NaOH so as to furnish a sharper end-point. For the sake of uniformity, however, all results are recorded in terms of $N/20$ NaOH. Edestin, leucylglycine (LG.) and glycylglycine (GG.) were again used as substrates, the former for the investigation of proteinase activity and the two latter for the investigation of dipeptidase activity.

The use of 40% formaldehyde adjusted colorimetrically to pH 8·5 calls for comment, more especially in those instances in which the quantity of amino-acid present in the titrated liquid is low, as, for example, in the reaction mixtures prepared during the first 2 days of the tests on the effect of germination time on proteinase activity (see below). The addition of the adjusted formaldehyde to an equal volume of reaction mixture after its titration to stage (*a*) sometimes resulted, not in the expected discharge of the pink colour, but in its intensification. Addition of the formaldehyde to an equal volume of water produced a similar result. After the discharge of the intensified pink colour by a known amount of $M/20$ potassium hydrogen phthalate (usually 1 ml.) back-titration with NaOH revealed the extent of the apparent development of alkalinity on dilution of the formaldehyde. An example may perhaps clarify this statement: 10 ml. of 40% formaldehyde (containing 10 drops phenolphthalein) adjusted colorimetrically to pH 8·5, after the addition of 10 ml. water, 1 ml. $M/20$ potassium hydrogen phthalate and 11 more drops of indicator (to maintain its concentration at 1 drop per ml.) required 0·24 ml. $N/20$ NaOH to restore the pink colour to that of the pH 8·5 standard. Deducting this latter value from that required by the $M/20$ potassium hydrogen phthalate (namely, 1 ml.) gives 0·76 ml. $N/20$ NaOH as the apparent development of alkalinity on mixing 10 ml. formaldehyde at pH 8·5 with 10 ml. water. This value will vary according to the accuracy of the preliminary colorimetric adjustment of the formaldehyde to pH 8·5 and with the amount of formaldehyde required in the titration. Thus all quantitative determinations of amino-acids by the above method require individual correction by the addition of the NaOH equivalent of the apparent development of alkalinity. Results obtained by difference between titres at the beginning and end of a given period are, of course, not subject to this correction providing the same procedure is observed throughout. Correction is necessary, however, in those instances in which the initial titre (*a*) is less than, and the final titre (*b*) is greater than, the apparent development of alkalinity figure (cf. initial stages of germination experiments).

The apparent development of alkalinity is, of course, due to the increase in the *p*K′ value of phenolphthalein in the 40 % formaldehyde. This alteration in *p*K′ commences at some point between 20 and 40 % concentration since below 20 % the *p*K is the same as in aqueous solution [Richardson, 1934].

RESULTS.

Effect of germination time on proteinase activity.

The germination of wheat seeds results in increase in proteinase activity: the rate of increase is shown in Fig. 1. 50 g. portions of the same wheat were allowed to germinate for varying lengths of time at 18°. The grains were first immersed in tap water at 18° for 24 hours, being afterwards transferred to perforated porcelain germinating trays and kept covered with moist absorbent cotton-wool. After the appropriate period each portion was minced finely and its gain in weight made up to 100 % with water. The mass was then extracted with 67 ml. water containing a few drops of toluene for 2 hours at 30° and filtered. The 24-hour immersion period was considered as part of the germination period. The initial value was obtained by the use of an extract prepared from 50 g. of finely ground wheat seeds. Germination was not continued beyond the seventh day as mould growth appeared after that time.

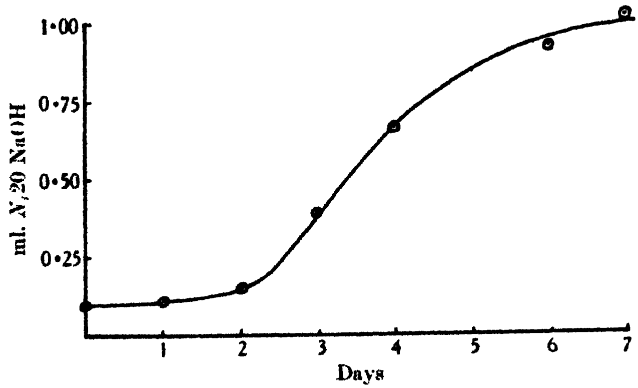

Fig. 1. Effect of germination time. Concentration of edestin in reaction mixture: 2·0 %. *M*/7 acetate buffer at *p*H 4·1. Enzyme dilution 2 : 7. Formaldehyde titration of 4-hour samples. Temperature 40°. Titres as ml. *N*/20 NaOH per 5 ml. sample in excess of a control solution without edestin.

Ageing of wheat seeds.

The effect of ordinary storage on dry wheat seeds is illustrated in Fig. 2. The curve is constructed from figures obtained over a period of approximately 2 years. A quantity of English wheat (1934 crop) was kept in a glass-stoppered bottle at 18° and in the dark. Samples were withdrawn at intervals, allowed to germinate for 5 days, and the proteolytic activity of an aqueous extract determined in the usual way with edestin as substrate.

Influence of glycerol on dipeptidase stability.

In the previous communication it was shown that the proteinase activity of aqueous extracts of sprouted wheat seeds diminishes to half its original value in about 18 days on storage in the dark at 18° and in the presence of toluene whereas the activity of the dipeptidase is reduced to zero in 5 days under similar conditions. The inclusion of glycerol in the wheat extract was found to increase considerably the stability of the dipeptidase (see Table I), activity being maintained almost at the original level even after 21 days at 18°. In Table I are included for convenience the results already recorded for the stability of the dipeptidase in aqueous extract. Extracts containing glycerol were prepared by replacing 200 ml. of the extraction water with an equal quantity of 98 % glycerol per 150 g. wheat used. The concentration of the glycerol in the extraction mixture was approximately 45 %. Other conditions remained unaltered. The colour of the glycerol extracts invariably remained a deep golden yellow in contrast to the aqueous ones which usually deepened to a dirty brown shade.

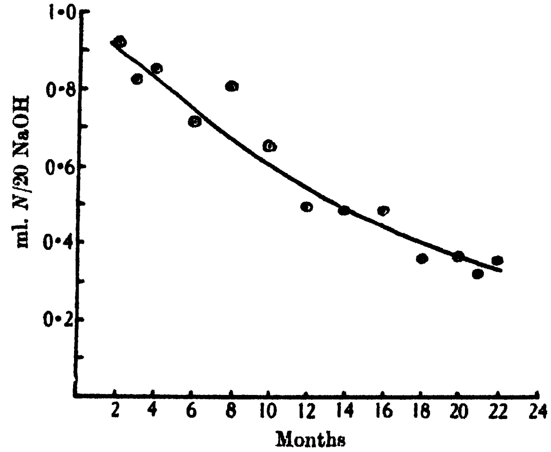

Fig. 2. Ageing of wheat seeds. Details as in Fig. 1.

Table I.

Days of storage	...	0	1	2	4	7	14	21
Increase in titre (*b*):	Aqueous	1·34	1·16	0·62	0·06	—	—	—
	Glycerol	1·12	1·08	1·14	1·10	1·13	1·10	1·08

Concentration of LG. in reaction mixture: $M/25$. Enzyme dilution 2 : 7.
Concentration of glycerol in reaction mixture: 14 %.
Titration of 3-hour samples. Reaction at *p*H 7·3.

The *p*H value of the aqueous extracts in the majority of cases fell rapidly on storage from about 6 to a value between 4 and 5. The glycerol extracts, however, did not vary in this manner: their acidity was either maintained at the original value of *p*H 6 or in some few cases tended even to rise slightly. Representative figures for aqueous and glycerol extracts are given in Table II. The *p*H values of the glycerol extracts were calculated from the millivolt readings in the usual manner, no allowance being made for any change in dielectric constant etc. by the inclusion of 14 % glycerol in the reaction mixture.

Table II.

Days of storage	...	0	1	2	4	7	14	21
*p*H value:	Aqueous	6·02	5·10	4·83	4·64	4·50	4·41	4·33
	Glycerol	6·05	—	6·12	6·23	6·40	6·52	6·64

Effect of pH *on stabilities of proteinase and dipeptidase.*

From the data presented in the previous section it appears probable that the increase in acidity on storage of aqueous extracts is responsible for the destruction of the dipeptidase. The stabilities of both proteinase and dipeptidase were therefore measured during the storage of aqueous extracts at various *p*H values.

Three separate portions of the same fresh aqueous extract were adjusted by means of acetate buffer or NaOH solution to *p*H 4, 6 and 8, respectively. The innate tendency of the extracts to develop acidity on standing necessitated the frequent readjustment of the alkaline extract to *p*H 8 by the cautious addition of dilute NaOH. At no time did the reaction of this extract vary by more than 0·2 unit from the desired figure. The two acid extracts maintained constant *p*H values. The extract adjusted to *p*H 4 remained a pale brown, slightly turbid liquid, whereas that at *p*H 6 rapidly darkened to a dirty brown shade. The extract at *p*H 8 remained throughout a fairly clear golden yellow. The activity of each extract was determined at intervals both on edestin at *p*H 4·1 and on leucylglycine at *p*H 7·3. The results are shown graphically in Figs. 3 and 4.

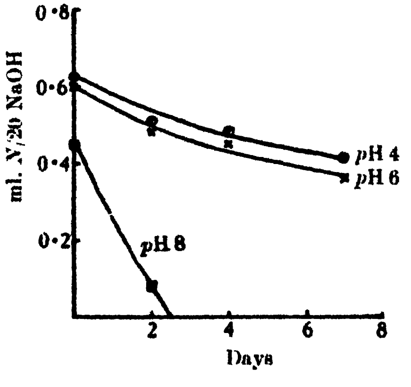

Fig. 3.

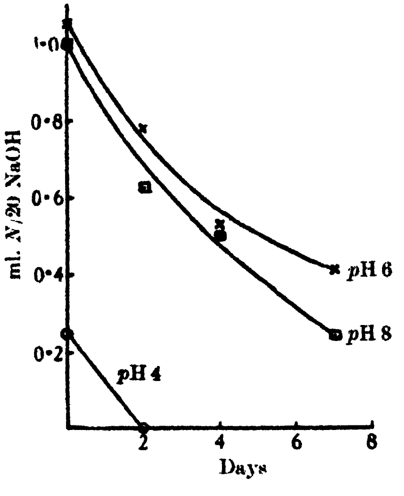

Fig. 4.

Fig. 3. Proteinase stability. Concentration of edestin in reaction mixture: 1·6 %. *M*/7 acetate buffer at *p*H 4·1. Enzyme dilution 2 : 7. Titration of 3-hour samples. ⊙ *p*H 4. × *p*H 6. ☐ *p*H 8.

Fig. 4. Dipeptidase stability. Concentration of LG.: *M*/25. Enzyme dilution 2 : 7. Reaction at *p*H 7·3. Titration of 3-hour samples. ⊙ *p*H 4. × *p*H 6. ☐ *p*H 8.

Activation of proteinase by cyanide.

The rate of hydrolysis of edestin by wheat proteinase is markedly accelerated in the presence of cyanide, the *p*H optimum at the same time being shifted towards the alkaline side from *p*H 4·1 to 4·8. In Fig. 5 are reproduced the *p*H optimum curves for the action of the proteinase on edestin in the presence and in the absence of cyanide. The latter substance was added to the reaction mixtures in the form of sodium cyanide.

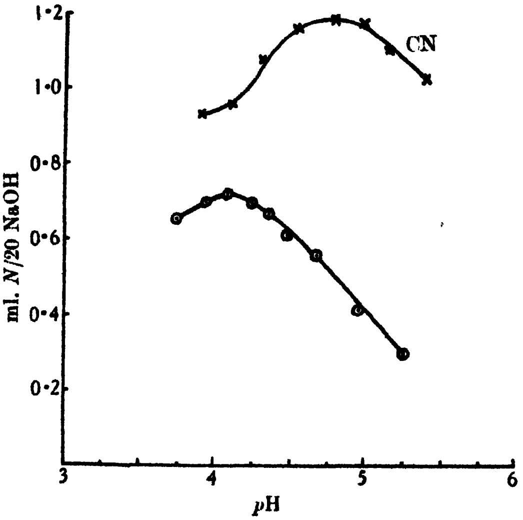

Fig. 5. Activation of proteinase by cyanide. Concentration edestin: 1·6 %. Concentration NaCN: 0·01 M. Enzyme dilution 2 : 7. $M/7$ acetate buffers. ⊙ Normal. × With CN.

Relation between cyanide concentration and proteinase activation. The effect of variation in cyanide concentration on the rate of hydrolysis by wheat proteinase is shown by the graph in Fig. 6. Reaction mixtures containing cyanide were maintained at the appropriate optimum, pH 4·8: the initial mixture containing no cyanide was adjusted to pH 4·1, the optimum in the absence of cyanide.

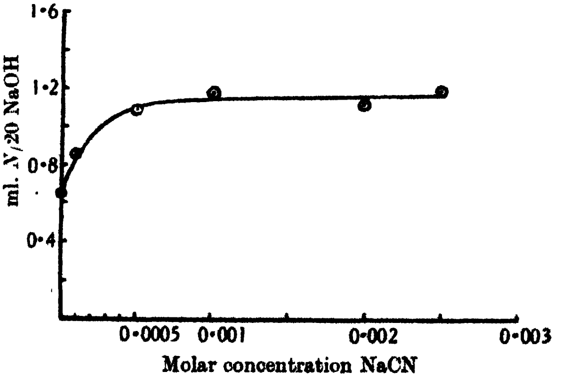

Fig. 6. Activation with varying cyanide concentration. Concentration edestin: 1·6 %. Enzyme dilution 2 : 7. $M/7$ acetate buffers.

Activation of dipeptidase by cyanide.

Cyanide also accelerates the action of wheat dipeptidase on both glycylglycine and leucylglycine. In both cases the optimum is shifted towards the alkaline side, to a greater extent with LG. as substrate than with GG. (Figs. 7 and 8.)

Discussion.

Germination studies on wheat in which particular reference has been made to the proteolytic enzyme content of the seeds have been reported by Bach & Oparin [1922], Bach *et al.* [1927] and Pett [1935]. Bach *et al.* found the proteinase activity of wheat to be increased about 40 times on germinating up to the eighth day. Enzymic activity was determined by precipitation of the amino-acids as their copper salts with subsequent estimation of the N content of the precipitate by Kjeldahl's method. Much of the work was unfortunately carried out without due regard to such factors as optimum *p*H, effect of drying etc. Pett's contribution was a study of the distributions of both proteinase and dipeptidase in five easily dissected parts of the seed, namely, hull, endosperm, scutellum, plumule and radicles. His observations showed clearly that although hull and endosperm possess little or no proteolytic activity either initially or on germination, scutellum and embryo are more liberally endowed with proteinase and dipeptidase even in the dormant state. On germination these latter two portions of the seed show a rapid increase in activity. In the scutellum this increase continues up to 36 hours and in the embryo up to 12 hours, activity afterwards diminishing. The present author's observations (see Fig. 1) show little significant increase in proteinase activity until the third day of germination, increases thereafter being rapid until about the sixth day. On the seventh day the activity is approximately ten times that in the dormant seed. The apparent differences between Pett's results and the present ones may possibly find an explanation in the methods by which the two sets of results were obtained. Pett's determinations were carried out on dissected portions of seeds whereas the present ones involved the extraction of whole germinated seeds and hence slight variations in the activities of the various parts would tend to be levelled out. The lag phase extending over the first 2 days may be occasioned by an incomplete diffusion of enzyme into solution during extraction owing to the imperfect opening up of the scutellum and embryo cells. By the third day of germination the condition of these latter portions may allow a better release of enzyme content on mincing and extracting. Support is gained for this view by the observa-

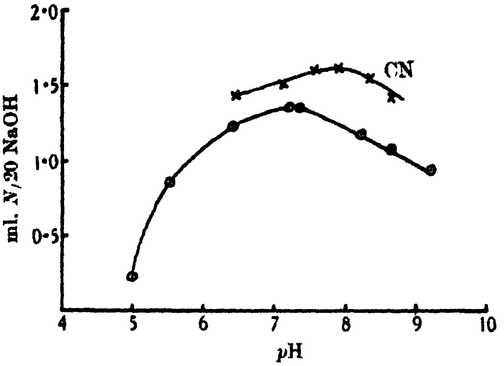

Fig. 7.

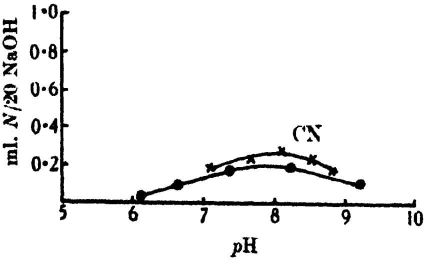

Fig. 8.

Fig. 7. Activation of dipeptidase by cyanide. Concentration LG. = $M/25$. Concentration NaCN: 0·01 M. Enzyme dilution 2 : 7. ◎ Normal. × With NaCN.

Fig. 8. Activation of dipeptidase by cyanide. Concentration GG. = $M/25$. Concentration NaCN: 0·01 M. Enzyme dilution 2 : 7. ◎ Normal. × With NaCN.

tion that the quantity of protein extracted during the process corresponded closely with the quantity of enzyme, being almost negligible during the first 2 days of germination, afterwards increasing rapidly up to the sixth or seventh day. The increase in enzymic activity on germination ran parallel with the physical changes in the seeds: the embryo developed slowly for the first 2 days and then showed a considerable increase in size on the third and fourth days. By the seventh day the acrospires had attained an average length of 2 in. and were tipped with green whilst the radicles were about 2½ in. long. Barley, on germination, underwent changes similar to those described above [Lüers, 1936].

The percentage germination of wheat seeds is usually taken as a measure of the vitality of the seeds. Under ordinary conditions of storage vitality persists only for about 10 years [Bradley, 1935] although under special conditions vitality has been demonstrated in seeds 20 years' old [Whymper, 1935]. It is reasonable to assume that loss of vitality is accompanied by loss of enzymic activity and the results depicted in Fig. 2 bear out this assumption. It is somewhat surprising however to find that in the comparatively short period of 2 years the power to develop proteinase activity on germination dwindled to about one-third of its initial value.

The stability of the dipeptidase of sprouted wheat extracts is enhanced very considerably by the presence of glycerol, no loss of activity being observed after 21 days in the presence of 45% glycerol whereas in its absence all activity disappeared after 5 days at 18° and in the presence of toluene. These observations are in close accord with those of Linderstrøm-Lang & Sato [1929]. The observation that glycerol extracts, unlike aqueous ones, did not develop acidity on storage (see Table II) led to the investigation of the effect on enzyme stability of storage of the extract at various *p*H values. From the results presented in Figs. 3 and 4 it is evident that the influence of *p*H during storage is considerable. The proteinase is comparatively stable at *p*H 4 and 6 but is rapidly destroyed at *p*H 8, whilst the dipeptidase remains quite active at *p*H 6 and 8, losing all activity however by a short exposure of less than 2 days to a medium of *p*H 4. Lüers & Malsch [1929] found the peptidase of green malt to be destroyed rapidly below *p*H 5. At *p*H 6 both wheat enzymes are fairly stable although the loss of activity by the dipeptidase occurs at an appreciably greater rate than that sustained by the proteinase. At *p*H 4 the wheat extract is deprived of four-fifths of its dipeptidase content within a few minutes. The proteinase is not so readily destroyed at *p*H 8: it still retains slight activity after 2 days. Storage of sprouted wheat extracts at *p*H 4 and 8, respectively, furnishes therefore an additional means of separation of proteinase from dipeptidase. After 2 days at 18° at *p*H 4 the extract is dipeptidase-free, whilst after 3 days at *p*H 8 the extract is proteinase-free. The results also provide further proof of the separate identities of the two enzymes.

From Fig. 4 it is clear that the increased stability of dipeptidase in presence of glycerol is due largely to the action of the latter in maintaining the acidity of the wheat extract at or about *p*H 6. The effect of the glycerol however is not merely to retard acid production since at *p*H 6 and even at *p*H 8 the dipeptidase still suffers an appreciable loss of activity in the comparatively short time of 7 days, whereas in the presence of glycerol the enzyme retains its original vigour for at least 21 days. The precise action of glycerol is not certain but it is probable that its main effect depends upon a lowering of the rates of the various reactions which undoubtedly proceed in an aqueous extract of sprouted wheat and which are probably responsible for the destructive effect. Hydrolytic processes in particular would almost certainly be retarded by concentrations of glycerol as high as 45 %. The absence of pigment formation and acid production in glycerol extracts supports this view.

Activation of plant proteinases by cyanide is a well-known phenomenon, papain [Willstätter & Grassmann, 1924], pineapple proteinase [Willstätter *et al.* 1926], yeast proteinase [Grassmann & Dyckerhoff, 1928, 2] and the proteinase of green malt [Lüers & Malsch, 1929] being among the principal enzymes affected in this way. The proteinase of sprouted wheat extract is also activated by cyanide, the increase in rate of reaction as shown in Fig. 5 being about 64 %. Actually the extent of activation varied between 50 and 64 % for different preparations of wheat extract. Lüers & Malsch reported a 50 % increase in the activity of green malt infusions in the presence of cyanide. Papain, on the other hand, showed a 200–300 % increase in activity. The *p*H optimum of the latter enzyme was unaffected by the addition of the cyanide although its specificity was widened. Cyanide however caused a shift in the respective *p*H optima of the proteinases of both green malt and sprouted wheat. Using gelatin as substrate Lüers & Malsch found an optimum of *p*H 4·9–5·0 for green malt proteinase: with cyanide the optimum was lowered slightly to *p*H 4·6 and 4·7. The effect of cyanide on sprouted wheat proteinase was to cause a shift in the optimum for its action on edestin from *p*H 4·1 to 4·8.

Fig. 6 shows also that the activation of the proteinase reaches a maximum at a cyanide concentration of about 0·001 *M*, the extent of activation thereafter remaining the same even up to 0·05 *M*, the highest concentration investigated.

The effect of cyanide upon the majority of peptidases so far tested in this way is one of inhibition, not of activation. Among those inhibited are erepsin [Euler & Josephson, 1926], the yeast peptidases [Grassmann & Dyckerhoff, 1928, 1] and yeast polypeptidase [Grassmann & Dyckerhoff, 1928, 2]. The dipeptidase of wheat, on the contrary, is activated when both LG. and GG. are used as substrates (Figs. 7 and 8). The extent of activation in both instances is less than that exhibited by the proteinase. For LG. the increase varied from 5 to 20 % with an accompanying shift in the optimum from *p*H 7·3 to about 7·8. For GG. an increase of about 10 to 30 % was recorded with a slight shifting of the optimum from *p*H 7·9 to 8·1.

SUMMARY.

1. The proteinase activity of germinating wheat seeds, after a lag phase of 2 days, increases tenfold in 7 days at 18°.

2. Wheat seeds, stored in the dark at 18°, display a steady loss of power to develop proteinase activity on germination, the loss amounting to about 67 % in 2 years.

3. The stability of the dipeptidase of sprouted wheat extract is raised considerably in the presence of 45 % glycerol; the activity remaining at the original level for at least 21 days whereas in aqueous solution all activity is lost in 5 days at 18°.

4. Wheat proteinase is relatively stable in buffer solutions maintained at *p*H 4 and 6 respectively but is destroyed at *p*H 8 at 18° in less than 3 days.

5. Wheat dipeptidase loses its activity comparatively slowly at *p*H 6 and 8 but is almost immediately destroyed by exposure to a medium of *p*H 4.

6. Glycerol doubtless owes much of its stabilizing power to its ability to maintain the acidity of sprouted wheat extracts at or about their initial value of *p*H 6: aqueous extracts almost invariably develop acidity, falling generally from *p*H 6 to between *p*H 4 and 5 in 1 or 2 days.

7. Both the proteinase and dipeptidase of sprouted wheat are activated by cyanide. The former enzyme is increased in activity by about 60%, suffers a shift in the optimum for its action on edestin from pH 4·1 to 4·8 and shows a maximum requirement of 0·001 M cyanide. The dipeptidase is stimulated to a less extent than the proteinase but shows similar shifts in pH optima towards the alkaline side. For leucylglycine the shift is from pH 7·3 to 7·8 and for glycylglycine from pH 7·9 to 8·1.

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OBITUARY NOTICE.

WILLIAM HOLDSWORTH HURTLEY.

1865–1936.

William Holdsworth Hurtley was born at Armley near Leeds in 1865 and was educated at the Yorkshire College, now the University of Leeds, from which he graduated in the University of London, taking the B.Sc. in 1888 and thirteen years later the D.Sc. After teaching for some years in a school at Leeds and at the University Tutorial College in Red Lion Square, London, he was appointed Demonstrator in the Medical College of St Bartholomew's Hospital in 1899 and in 1906 succeeded to the Lectureship. This position he had decided to resign at the close of the summer session when in February he fell ill with bronchitis, from which he had many times suffered, and died in the Hospital on June 2nd.

A funeral service was held in St Bartholomew-the-Less when the church was completely filled by his colleagues and students. He leaves a widow, a son and two daughters.

Hurtley was a member of the Board of Studies in Chemistry and for many years acted as one of the Internal Examiners in the University of London of which he became a Reader in 1919. He also several times examined for the Conjoint Board. He was elected a Fellow of the Chemical Society in 1912 and was an original member of the Biochemical Society.

During the whole of his long connection with the Medical School of St Bartholomew's Hurtley endeared himself to all with whom he came into contact by his integrity, modesty and invariable readiness to assist any colleague or student. He was an excellent teacher and lecturer and although his duties, especially in recent years, were unduly arduous he carried out much useful and important research. This was at first purely chemical, but from his association with a great hospital his attention was soon directed to the more clinical aspects of biochemistry, much of his work being concerned with the detection and estimation of abnormal substances in urine.

His researches include, "A critical examination of methods for the estimation of potassium and sodium in urine and other material of organic origin" (Hurtley & Orton, *J. Physiol.* 1904, **30**, 10), and papers dealing with the isolation of products obtained by the benzoylation of urine from cases of cystinuria (Garrod & Hurtley, *J. Physiol.* 1906, **34**, 317) and with the supposed occurrence of uroleucic acid in the urine from some cases of alkaptonuria (Garrod & Hurtley, *J. Physiol.* 1907, **36**, 136). These were followed by interesting papers on the distillation of butter fat, coconut oil and their fatty acids (Caldwell & Hurtley, *Trans. Chem. Soc.* 1909, 853) and on the interaction of alloxan and glycine (Hurtley & Wootton, *Trans. Chem. Soc.* 1911, 288). In 1913 he published an excellent survey of the methods of detecting acetoacetic acid in urine, including a description of a new, very delicate test depending on the formation of a highly coloured ferrous salt of *iso*nitrosoacetone, the depth of the colour giving a fairly accurate quantitative value (Hurtley, *Lancet*, 1913, i, 1160). This paper was followed by one dealing with the physiological action of acetoacetic acid and related substances (*J. Physiol. Proc.*, July 15, 1916).

His most important piece of work however was upon the 4-carbon acids of diabetic urine, an account of which was published in *Quart. J. Med.* 1915–16, **9**, 301–408. In this paper which records an immense amount of very accurate and painstaking work, the effect of different diets on the excretion by diabetic patients of β-hydroxybutyric and acetoacetic acids is described and the toxicity of the latter and their origin from fats discussed with admirable lucidity.

This paper was followed by one on the estimation of calcium (Cahen & Hurtley, *Biochem. J.* 1916, **10**, 308) and another entitled "The oxidation of the alkali butyrates by hydrogen peroxide with the production of succinic acid (Cahen & Hurtley, *Biochem. J.* 1917, **11**, 164) in which it was shown that in the oxidation of β-hydroxybutyric acid more than half was converted into succinic acid, the formation of which Dakin (*J. biol. Chem.* 1908, **4**, 77), who had previously studied the reaction, had failed to observe.

Hurtley's work after this time was much interrupted by the great influx of medical students on the conclusion of the war but he published a few years ago (Allott, Dauphinee & Hurtley, *Biochem. J.* 1932, **26**, 1665) a paper in which he described a modified procedure for the estimation of iodine in blood, upon which has been based an improved and standardized method for determining small quantities of iodine in such substances as blood, milk and vegetable food (*The Determination of Iodine in Biological substances*, His Majesty's Stationery Office, 1935). For some time before his death Hurtley was occupied with the separation and purification of the bile acids and working on comparatively small quantities of ox-bile, had devised a method by which increased yields of nearly all the acids known to occur in it could be obtained in a high state of purity. He was also writing a monograph on the bile acids for Messrs Longmans, Green & Co., which it is to be hoped he has left in such a state that it may be completed for publication.

F. D. C.

CCL. THE PRODUCTION OF MUCUS DURING THE DECOMPOSITION OF PLANT MATERIALS.

III. THE EFFECT OF PARTIALLY AEROBIC AND ANAEROBIC CONDITIONS.

By JAGESHWAR GOPAL SHRIKHANDE.

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(*Received 9 June 1936.*)

THE conditions necessary for the production of stickiness in manures described in the first two parts [1933, 1, 2] of this series were studied chiefly under aerobic conditions. While conducting experiments on the applicability of the Edelmist process to the composting of town refuse under tropical and sub-tropical conditions, it was thought worth while to follow the production of stickiness under partially aerobic conditions. Ragi straw, *Eleucine coracana*, was therefore decomposed in the presence of different sources of available nitrogen, the carbon-nitrogen ratios being adjusted to 30 : 1 and 10 : 1. The amount of mucus produced was measured by the physical test described in Part I [1933, 1]. No attempt was made to analyse the different structural constituents of the straw as no correlation was found to exist between the rates of their disappearance and the formation of mucus [1933, 2].

EXPERIMENTAL.

The physical test was conducted on fermented straws obtained under conditions described below.

Ragi straw was rotted in the presence of a mixed natural flora at 35° with the following changes in aeration, intervals of time and adjustment of reaction.

(1) Initial aerobic fermentation of 14 days followed by loose packing in bottles and analysing at intervals of 14, 28 and 56 days.

(2) As in (1) except that the bottles were tightly packed.

(3) Completely anaerobic conditions throughout the experiment, attained by packing air-tight in bottles and analysing at intervals of 14, 28 and 56 days.

(4) Completely aerobic conditions throughout the experiment and analysing on the 28th and 56th days after commencement.

(5) Adjustment of the final *p*H to a constant value independent of the source of nitrogen.

TECHNIQUE.

Ragi straw was chaffed and weighed quantities were fermented aerobically in large bottles with different amounts of nitrogen in various forms. The moisture content was maintained at about 60 %. After 14 days the contents were weighed and distributed into small bottles for subsequent decomposition. Weighed quantities of the thoroughly mixed material were taken and packed into bottles as required. The bottles were fitted with paraffined corks carrying exit tubes and pinch-cocks. The enclosed air for experiments in series II and III was sucked away and the bottles were then incubated at 35°. The pinch-cocks were opened at intervals of 3 days to let out the accumulated gases. This process had to be repeated more frequently when night soil was used as a source of available nitrogen, the fermentation being very vigorous in the first 4 or 5 days. At the end of the desired period the bottles were opened and weighed and the contents subjected to stickiness test.

Table I. *Loss of dry matter per 100 g. of original straw and variation in stickiness under different conditions of fermentation.*

Series I unrestricted air supply (U) followed by partially restricted air supply (PR).

Time in days	Loss of dry matter	Physical test g.	Time in days	Loss of dry matter	Physical test g.
	$(NH_4)_2SO_4$, 30 : 1.			$NaNO_3$, 30 : 1.	
U 0	0	—	U 0	0	—
14	14·3	437	14	16·9	4444
PR 28	13·9	107	PR 28	17·2	3009
42	15·2	0	42	27·0	3140
70	26·2	0	70	33·1	2856
	$(NH_4)_2SO_4$, 10 : 1.			$NaNO_3$, 10 : 1.	
U 0	0	—	U 0	0	—
14	3·6	0	14	6·2	2069
PR 28	7·5	0	PR 28	13·9	1096
42	11·5	0	42	19·6	2778
70	19·9	0	70	27·8	1997
	$(NH_4)_2CO_3$, 30 : 1.			Night soil, 30 : 1.	
U 0	0	—	U 0	0	—
14	11·7	2190	14	24·7	5450
PR 28	16·9	1876	PR 28	25·7	3000
42	18·8	869	42	29·3	1076
70	27·7	798	70	43·7	1133
	$(NH_4)_2CO_3$, 10 : 1.			Night soil + $(NH_4)_2SO_4$, 10 : 1.	
U 0	0	—	U 0	0	—
14	3·6	0	14	19·8	4370
PR 28	12·9	2049	PR 28	16·7	987
42	19·9	775	42	21·1	923
70	27·3	810	70	24·5	0

RESULTS.

Series 1. Variation in stickiness of manures obtained by a preliminary aerobic followed by partially aerobic fermentation. Table I contains figures for stickiness and losses of dry matter obtained with such fermentation. The losses of dry matter after 14 days of aerobic decomposition are comparable with those previously recorded but the rate of decomposition appears to slow down as the period of restricted supply of air increases. The maximum losses occur with sodium nitrate and night soil as the sources of available nitrogen. In both of these cases the pH was higher than with ammonium sulphate or carbonate. The decomposition, therefore, depends upon the final reaction of the manure. Stickiness also runs parallel with the losses of dry matter and the final reaction of the manure. That there exists a relationship between the reaction and stickiness can be more clearly seen from the last two sections of Table I. Night soil with ammonium sulphate has produced less stickiness than night soil alone.

Table II. *Loss of dry matter per 100 g. of original straw and variation in stickiness under different conditions of fermentation.*

Series II unrestricted air supply (U) followed by completely restricted air supply (CR).

Time in days		Loss of dry matter	Physical test g.	Time in days		Loss of dry matter	Physical test g.
		$(NH_4)_2SO_4$, 30 : 1.				$NaNO_3$, 30 : 1.	
U	0	0	—	U	0	0	—
	14	13·9	564		14	18·9	6459
CR	28	27·8*	678	CR	28	24·0	2897
	42	20·0	107		42	27·3	676
	70	24·7	110		70	29·6	208
		$(NH_4)_2SO_4$, 10 : 1.				$NaNO_3$, 10 : 1.	
U	0	0	-	U	0	0	—
	14	14·8	459		14	10·5	2875
CR	28	23·1	203	CR	28	23·4	2060
	42	26·7	0		42	25·9	555
	70	29·7	0		70	29·1	210
		$(NH_4)_2CO_3$, 30 : 1.				Night soil, 30 : 1.	
U	0	0	—	U	0	0	—
	14	23·1	2871		14	21·9	3597
CR	28	24·1	708	CR	28	23·2	1050
	42	27·0	171		42	23·7	1025
	70	30·8	0		70	28·4	0
		$(NH_4)_2CO_3$, 10 : 1.				Night soil + $(NH_4)_2SO_4$, 10 : 1.	
U	0	0	—	U	0	0	—
	14	10·5	2344		14	18·7	3049
CR	28	19·8	1019	CR	28	28·2	889
	42	22·5	0		42	30·8	114
	70	27·5	0		70	32·1	506

* Gone aerobic.

Incorporation of ammonium sulphate lowers the *p*H and the greater acidity reduces the stickiness. Sodium nitrate and night soil give the maximum stickiness. The stickiness obtained on aerobic fermentation gradually disappears as the supply of air gets less. This is perhaps due to the amounts of organic acids liberated during partially anaerobic conditions. The bottles when opened smelt of lactic acid. These points are more clearly brought out in the following series.

Series II. Variation in stickiness of manure obtained by a preliminary aerobic followed by anaerobic fermentation. The contents of the bottles were highly foul-smelling and smelt of organic acids, mostly acetic and lactic. The decomposition is practically of the same order though the rate is lower than in Series I. The values for stickiness recorded in Table II are also much lower than those in Table I and the rate of fall in stickiness is greater as the period of anaerobic decomposition increases. Such differences both in the losses of dry matter and stickiness can safely be attributed to the total absence of air. More acids are generated during anaerobic fermentation, which destroy the organisms responsible for a satisfactory rot and the production of stickiness. Sodium nitrate and night soil maintain some stickiness even at the end of 70 days because of their comparatively high final *p*H. It is well established that under anaerobic conditions nitrates are rapidly denitrified in presence of organic matter, leaving a strong base which serves as a neutralizing agent. Night soil too during fermentation in the early stages gives out ammonia which to a considerable extent neutralizes these acids.

Series III. Variation in stickiness of manure obtained under entirely anaerobic conditions. The losses of dry matter and the rate of decomposition as seen in Table III are extremely poor when compared with the first two series. The manure was also non-sticky. Results in Table III, when compared amongst themselves, show that the maximum loss of dry matter was obtained with sodium nitrate and night soil owing to the reaction being more nearly neutral. The contents of the bottles were not so foul-smelling as in Series II, but smelt strongly of acetic acid. The colour was also peculiarly pale yellow, thus differing from that in the foregoing series which was distinctly brown. The complete absence of stickiness must, therefore, be ascribed to anaerobic conditions preventing the growth of those aerobic types of fungi and bacteria which are mostly responsible for the production of stickiness, as has been suggested in Part II [1933, 2].

Table III. *Loss of dry matter per 100 g. of original straw and variation in stickiness under different conditions of fermentation.*

Series III. Completely restricted supply of air throughout the experiment (CR).

Time in days	Loss of dry matter	Physical test g.	Time in days	Loss of dry matter	Physical test g.
	$(NH_4)_2SO_4$, 30 : 1.			$NaNO_3$, 30 : 1.	
CR 0	0	—	CR 0	0	—
14	6·9	0	14	3·3	0
28	7·5	0	28	5·0	0
56	21·9*	110	56	6·9	0
	$(NH_4)_2SO_4$, 10 : 1.			$NaNO_3$, 10 : 1.	
CR 0	0	—	CR 0	0	—
14	14·7	0	14	15·3	593
28	17·0	0	28	10·5	471
56	16·9	0	56	16·9	0
	$(NH_4)_2CO_3$, 30 : 1.			Night soil, 30 : 1.	
CR 0	0	—	CR 0	0	—
14	10·8	0	14	12·8	116
28	9·5	0	28	14·6	107
56	16·4	0	56	16·2	0
	$(NH_4)_2CO_3$, 10 : 1.			Night soil + $(NH_4)_2SO_4$, 10 : 1.	
CR 0	0	—	CR 0	0	—
14	21·5*	670	14	14·5	130
28	14·1	0	28	14·5	110
56	13·5	0	56	19·5	0

* Gone aerobic.

Series IV. Variation in stickiness of manure obtained under entirely aerobic conditions. This series was conducted by way of comparison. It has been discussed in more detail in Part I [1933, 1]. As previously recorded sodium nitrate gives the stickiest manure, followed by night soil and ammonium carbonate. On comparing all the results of the first three series with those in Table IV it will be clear that manure obtained under entirely aerobic conditions is the stickiest, whilst manure is absolutely non-sticky when obtained under anaerobic conditions. Presence of a plentiful supply of air is, therefore, an essential condition for the production of stickiness in a manure heap.

Series V. The effect of the modification of the final pH *on the production of stickiness obtained under various conditions detailed above.* The *p*H in all the

Table IV. *Loss of dry matter per 100 g. of original straw and variation in stickiness under unrestricted supply of air (U)—Series IV.*

Time in days		Loss of dry matter	Physical test g.	Time in days		Loss of dry matter	Physical test g.
		$(NH_4)_2SO_4$, 30 : 1.				$NaNO_3$, 30 : 1.	
U	14	23·0	200	U	14	28·9	4120
	56	39·4	270		56	37·5	7353
		$(NH_4)_2SO_4$, 10 : 1.				$NaNO_3$, 10 : 1.	
U	14	14·8	185	U	14	10·5	2689
	56	39·0	210		56	37·1	7244
		$(NH_4)_2CO_3$, 30 : 1.				Night soil, 30 : 1.	
U	14	23·1	3450	U	14	41·9	5978
	56	47·4	4500		56	54·3	6010
		$(NH_4)_2CO_3$, 10 : 1.				Night soil + $(NH_4)_2SO_4$, 10 : 1.	
U	14	20·5	3252	U	14	18·7	4856
	56	54·9	4553		56	56·0	5873

bottles fell considerably during the course of fermentation. Under restricted supply of air, ammonium sulphate gave the lowest and sodium nitrate the highest *p*H. The present series was undertaken to demonstrate the effect on stickiness of artificially raising the *p*H to 9·0 by adding sodium carbonate. This *p*H of 9·0 is usually attained by a sodium nitrate rot which has lost about 40 % of dry matter in about 5 weeks and is accompanied by a maximum stickiness of nearly 7000 g. Table V includes the results of such experiments. No appreciable increase in the stickiness after adjustment of the *p*H to 9·0 in any of the samples is noticeable. This must be due to the lower losses of dry matter and also to the entirely different nature and course of decomposition under partially aerobic and anaerobic conditions.

Table V. *Effect of the modification of* pH *on the production of stickiness.*

(*p*H adjusted to 9·0 with Na_2CO_3.)

	Source of nitrogen	Loss of dry matter	Final *p*H	Physical tests g. Before *p*H adjustment	Physical tests g. After *p*H adjustment
From series I Ratio 30 : 1	$(NH_4)_2SO_4$	13·9	4·8	107	203
	$(NH_4)_2CO_3$	18·8	5·2	869	997
	$NaNO_3$	27·0	6·8	3140	3808
	Night soil	29·3	6·2	1076	1300
From series II Ratio 30 : 1	$(NH_4)_2SO_4$	20·0	4·5	107	276
	$(NH_4)_2CO_3$	27·0	5·1	171	549
	$NaNO_3$	27·3	6·4	676	1050
	Night soil	23·2	6·2	1050	1529
From series III	$(NH_4)_2SO_4$, 30 : 1	—	4·5	—	—
	$(NH_4)_2CO_3$, 30 : 1	—	5·1	—	—
	$NaNO_3$, 10 : 1	10·5	6·0	417	500
	Night soil, 30 : 1	14·6	6·0	107	110

DISCUSSION.

The experiments described above suggest that the decomposition of plant tissues is much slower under anaerobic than under aerobic conditions. The losses in dry matter with narrow C : N ratio are in general more than those recorded with wide C : N ratio. This observation is in accord with those of previous workers. Organic acids are generated during anaerobic fermentation the accumulation of which is responsible for the retardation of fermentation and destruction of stickiness. Fowler & Joshi [1920] observed depressed fermentation of cellulosic materials when the acid concentration reached a level of 1 %. Experiments on anaerobic decomposition of rice straw by Acharya [1935] and the author's own results [1933, 1] on aerobic decomposition of oat straw show that the change is influenced by the reaction during fermentation. A *p*H of 7·5–8·0 is found to be suitable for a satisfactory rot. Non-production of stickiness under partially restricted and totally restricted supply of air must be due partly to the strongly acid reaction and partly to the different nature and course of the decomposition. Anaerobic conditions modify the flora completely. Essentially aerobic fungi and mucus-producing bacteria like *Spyrochaeta cytophaga*, which have been described as essential in Part II [1933, 2] for the production of stickiness, do not develop under anaerobic conditions. Consequently there is no synthesis of the material responsible for stickiness because of the possible absence of interaction between fungal tissue and the gum from a bacterium. For a manure heap to be sticky, therefore, it must be maintained alkaline and should be kept thoroughly aerobic. This will ensure a satisfactory rot with a good growth of fungi and mucus-producing bacteria leading to the synthesis of the material responsible for stickiness.

SUMMARY.

1. Decomposition of Ragi straw, *Eleucine coracana*, under different conditions of fermentation in presence of different sources of available nitrogen has been studied from the point of view of stickiness. The results obtained confirm those previously recorded [1933, 1, 2].

2. Strong acidity and a different course and nature of decomposition under partially aerobic and anaerobic conditions prevent the development of stickiness during fermentation.

3. Wide C : N ratio produces a greater loss than narrow C : N ratio. High doses of nitrogen appear to have no effect on the production of stickiness.

4. Final adjustment of the *p*H to 9·0 with sodium carbonate has practically no effect on the stickiness obtained during fermentation.

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CCLI. SPECTROPHOTOMETRY OF PROTEINS.

I. ABSORPTION SPECTRA OF TYROSINE, TRYPTOPHAN AND THEIR MIXTURES.

II. ESTIMATION OF TYROSINE AND TRYPTOPHAN IN PROTEINS.

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(Received 27 May 1936.)

I. ABSORPTION SPECTRA OF TYROSINE, TRYPTOPHAN AND THEIR MIXTURES.

MEASUREMENTS of the absorption spectra of the aromatic amino-acids have been frequently reported in the literature but the results are not in good agreement. For purposes evident in Part II it was required to know the absorption curves of tyrosine and tryptophan with accuracy. Their absorption curves were therefore redetermined. The method of Vierordt for estimating spectrophotometrically the concentration of the components of a mixture was also investigated in its application to mixtures of tyrosine and tryptophan.

Method.

Absorption measurements were made throughout on a Hilger Medium Quartz spectrograph E 316 (the dispersion of which is 18·6 cm. from 200 to 400 mμ) with Spekker photometer, using a condensed spark between tungsten steel electrodes as light source. The spectrograph was fitted by the makers with a wave-length scale which was checked at intervals with the mercury arc. The maximum error of the scale between 230·2 and 333·9 mμ was found to be + and −0·2 mμ. When exposing a plate on the spectrophotometer the wave-length scale was printed at the top and the bottom of the plate. The matching was made by eye as follows. The plate to be matched was placed gelatin face downwards on a piece of glass with a fine cursor line engraved on it at right angles to the spectral strips. Owing to the small play which must be allowed for the plate in the plate holder the spectra on two plates are not always similarly orientated with respect to the edge of the plate. An adjustor was therefore made by which the plate could be rotated slightly and the cursor line made to cut the wave-length scales at identical readings. The spectra were then viewed through a low-power microscope, the plate being moved so that the point at which each pair of spectra appeared to match lay on the cursor line. The wave-length at which the cursor line cut the scales was then read off. This is a more laborious method than the usual one of placing an ink mark below the match point but was adopted in order that several sets of matchings could be made on the same plate without bias.

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[1] Owen Williams Research Scholar.

Since it is well known that ions of acids and bases often exhibit marked differences in absorption as compared with the unionized forms, the absorption spectra were measured at values of *p*H removed at least 2 units from the *p*K values of the amino-acids. The values for the dissociation constants of tyrosine are well established, those of Neuberger [1934] have been taken; for tryptophan those of Schmidt *et al.* [1929].

	COOH *p*K	$-NH_2$ *p*K	—OH *p*K
Tyrosine	2·35	9·16	10·41
Tryptophan	2·38	9·39	—

The imino-nitrogen of tryptophan has extremely weak acidic and basic properties but the dissociation constant is outside the range of useful measurement.

Accordingly measurements of absorption were made on solutions in water, in *N*/10 alkali and in *N*/10 HCl.

Amino-acids. Tyrosine was obtained from three sources, (*a*) isolated from silk according to Morrow [1927]; (*b*) a gift from Mr Philpot; (*c*) a commercial sample of Hoffmann La Roche. These all gave nitrogen values by Kjeldahl estimation between 7·72 and 7·73 % (theoretical 7·73). Their absorption curves were

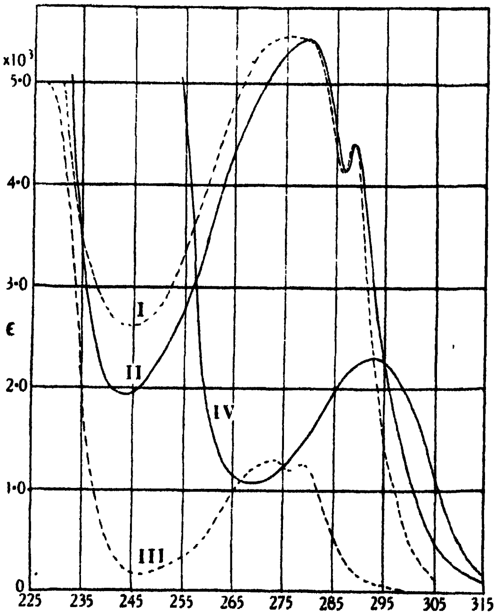

Fig. 1. I. Tryptophan in *N*/10 HCl. II. Tryptophan in *N*/10 NaOH. III. Tyrosine in *N*/10 HCl. IV. Tyrosine in *N*/10 NaOH.

Table I. *Molecular extinction coefficients of tyrosine and tryptophan.*

In *N*/10 NaOH $\epsilon \times 10^{-3}$.

Wave-length mμ ...	260	265	270	275	280	285	290	295	300	305	310	315
Tyrosine	1·75	1·14	1·08	1·24	1·54	1·96	2·24	2·26	1·89	1·14	0·471	0·182
Tryptophan	3·48	4·33	4·90	5·30	5·43	4·57	4·13	2·17	1·14	0·500	0·221	0·141

In *N*/10 HCl $\epsilon \times 10^{-3}$.

Wave-length mμ ...	260	265	270	275	280	285	290	295	300	305	310	315
Tyrosine	0·56	1·00	1·21	1·24	1·23	0·55	0·16	0·07	0·02	—	—	—
Tryptophan	4·07	4·84	5·33	5·47	5·43	4·33	3·67	1·33	0·50	0·13	—	—

identical within the limits of error of the method. Three samples of tryptophan were used, (*a*) a gift from Mr B. C. G. Knight, (*b*) a gift from Dr K. G. Stern of a sample of Fraenkel and Landau, (*c*) a preparation of Hoffmann la Roche. The absorption curve of tryptophan seems to be very sensitive to the presence of impurities. The two commercial samples required recrystallization twice from dilute alcohol before their absorption curves agreed with the sample of Mr Knight. Further purification yielded no change in the absorption spectrum. It is probable that the impurities in the commercial samples were decomposition products of the tryptophan as they were both some years old. These finally gave colour values according to Folin and Ciocalteau [1927] between 88·4 and 88·9 % by weight of that given by tyrosine (theoretical 88·7).

The absorption curves of the amino-acids are given in Fig. 1. The actual values for tyrosine and tryptophan are summarized in Table I.

Determination of tyrosine and tryptophan in mixtures of the two.

It is possible to estimate the concentrations of two components of a mixture knowing the absorption curves of standard solutions of each component and the absorption curve of the mixture provided that they do not interact. The method is due to Vierordt [quoted by Twyman & Allsopp, 1934].

If E_a and E_b be the extinction coefficients of the mixture at wave-lengths a and b,

ϵ_{1a} and ϵ_{2a} be the molecular extinction coefficients of components 1 and 2 respectively at λa,

ϵ_{1b} and ϵ_{2b} be the molecular extinction coefficients of components 1 and 2 respectively at λb,

M_1 and M_2 be the molar concentrations of components 1 and 2 respectively in the mixture,

then it can be shown that

$$M_1 = \frac{E_a \epsilon_{2b} - E_b \epsilon_{2a}}{\epsilon_{1a}\epsilon_{2b} - \epsilon_{2a}\epsilon_{1b}}, \qquad \ldots\ldots(1)$$

$$M_2 = \frac{E_b \epsilon_{1a} - E_a \epsilon_{1b}}{\epsilon_{1a}\epsilon_{2b} - \epsilon_{2a}\epsilon_{1b}}. \qquad \ldots\ldots(2)$$

Given a solution of a mixture of, say, tyrosine and tryptophan it is therefore necessary to measure the extinction coefficients of this mixture at two wave-lengths and to know the extinction coefficients of solutions containing known weights of tyrosine and tryptophan. From considerations discussed below it has been found that the most suitable wave-lengths are 280 and 305$m\mu$ to be measured on solutions of tyrosine and tryptophan in alkaline solutions whose pH is $>12{\cdot}0$.

Substituting in equations (1) and (2) the values given in Table I there result

$$M_{\text{tyrosine}} = (1{\cdot}0 E_{305} - 0{\cdot}092 E_{280}) \times 10^{-3},$$
$$M_{\text{tryptophan}} = (0{\cdot}21 E_{280} - 0{\cdot}288 E_{305}) \times 10^{-3}.$$

A test on a series of mixtures of tyrosine and tryptophan is reported in Table II.

Errors of the spectrographic method and of the analysis of mixtures.

The accuracy of the photographic method of spectrophotometry as here employed depends on the reproducibility of blackening of neighbouring small areas of a photographic plate as a result of equal exposures. This has been shown to be 5 % of the light intensity under favourable conditions [cf. Weigert, 1927].

This corresponds to an absolute error in E of 0·02. Von Halban & Eisenband [1927] put the error higher at 0·04 E. Besides this error there is that of matching. To assess this, 32 matchings were made at each of two points on a typical plate chosen at random. Some time was allowed to elapse between matchings to avoid eye strain or any possibility of remembering the spectral structure of those regions. Matchings were made alternately with the plate one way up and then inverted. It was found that there was a systematic difference between matchings made with the two orientations of the plate, the magnitude of which was very different with different observers, being large with the inexperienced and becoming almost negligible with practice. The eye appears to give preference of blackening to the lower of the pair of spectra in view. It is for this reason always advisable to match a plate in both ways. It was found that for the 32 matchings the standard deviation (σ) at 281·7 mμ was 0·288 mμ and at 301·6 mμ was 0·259 mμ which in each case corresponded to a standard deviation in extinction (σE) of 0·005E. Taking an arbitrary value of $3 \times \sigma E$ as the maximum error then this error of matching is $\pm 0{\cdot}015E$. It may therefore be conservatively stated that the error of the visual match point method of spectrography is less than $\pm 0{\cdot}055E$ for a single matching. By ensuring that the E measured is as high as possible the relative error is reduced. With the Spekker photometer densities up to 2·0 can be measured and it has been the practice in this work to arrange the concentration and thickness of the absorbing layer so that the density at the desired wave-length shall be greater than 1·0. It was more usually $> 1{\cdot}5$ and in the cases of the measurements given in Table I was always so. The values there given are the average of 6 matchings on each of 4 plates, i.e. 24 matchings for each point, and have a standard error of 0·003E.

The manner in which errors in measurement of the extinction coefficients of a mixture and in the values for the molecular extinction coefficients of the components are transmitted to the values obtained for the concentrations of the components in the mixture has been calculated by Mr J. St L. Philpot (private communication) to whom I am grateful for the following solution. It can be shown that in equations (1) and (2), the variances of M_1, i.e. ($\sigma^2 M_1$), and of M_2, i.e. ($\sigma^2 M_2$), are determined as follows:

$$\frac{\sigma^2 M_1}{M_1^2} = \frac{\left[2\left(\frac{M_2}{M_1}\right)^2 + \alpha^2 + \beta^2\right](\sigma^2_E + \sigma_\epsilon^2) + 2\frac{M_2}{M_1}(\alpha+\beta)\,\sigma^2_E}{(\alpha-\beta)^2}, \qquad \ldots\ldots(3)$$

$$\frac{\sigma^2 M_2}{M_2^2} = \frac{\left[2\left(\frac{M_1}{M_2}\right)^2 + \xi^2 + \eta^2\right](\sigma^2_E + \sigma_\epsilon^2) + 2\frac{M_1}{M_2}(\xi+\eta)\,\sigma^2_E}{(\xi-\eta)^2}, \qquad \ldots\ldots(4)$$

where $\alpha = \frac{\epsilon_{1a}}{\epsilon_{2a}} = \frac{1}{\xi}, \quad \beta = \frac{\epsilon_{1b}}{\epsilon_{2b}} = \frac{1}{\eta},$

and $\sigma^2_{E_{a \text{ or } b}} = \sigma^2_E$ and $\sigma^2_{\epsilon_{1a},\,\epsilon_{1b},\,\epsilon_{2a},\,\epsilon_{2b}} = \sigma^2_E$.

It follows that errors in M_1 and M_2 are more dependent on errors in E than on errors in ϵ. Further, in order that the errors in M_1 and M_2 shall be small it is required that $(\alpha-\beta)^2$ and $(\xi-\eta)^2$ shall be large. This is fulfilled in the case where the wave-lengths a and b are chosen on opposite sides of that wave-length at which the absorption curves of the two components cross one another. It will follow that if α is > 1 then ξ and β will be < 1 and $\eta > 1$. The curves of tyrosine and tryptophan in acid solution do not cross at any point but the curves of equimolar solutions in alkali cross at 294·5 mμ. The two wave-lengths 280 mμ and 305 mμ have been chosen to give the maximum reversal in the value of ϵ_1/ϵ_2 consistent with accurate measurement.

From Table I

$$\alpha=0{\cdot}284, \qquad \beta=2{\cdot}31,$$
$$\xi=3{\cdot}53, \qquad \eta=0{\cdot}432.$$

As an example of the application of this to the analysis of mixtures the case of a mixture of tyrosine and tryptophan in the molar ratio 3 : 1 is taken.

Substituting for α, β, ξ and η in equations 3 and 4 we get

$$\frac{\sigma^2 M_{\text{tyrosine}}}{M^2_{\text{tyrosine}}}=1{\cdot}8\sigma_E{}^2+1{\cdot}37\sigma_\epsilon{}^2,$$

and

$$\frac{\sigma^2 M_{\text{tryptophan}}}{M^2_{\text{tryptophan}}}=5{\cdot}8\sigma_E{}^2+3{\cdot}21\sigma_\epsilon{}^2.$$

σ_ϵ has been estimated as 0·003 and σ_E as 0·012. Inserting these values

$$\frac{\sigma M_{\text{tyrosine}}}{M_{\text{tyrosine}}}=0{\cdot}014 \qquad \frac{\sigma M_{\text{tryptophan}}}{M_{\text{tryptophan}}}=0{\cdot}026$$

giving an error of about ±4% of tyrosine and ±8% of the tryptophan concentrations.

As an example may be quoted the findings of an actual experiment. Two solutions were made up in $N/10$ NaOH: (*a*) tyrosine $0{\cdot}785\times10^{-3}\,M$, (*b*) tryptophan $0{\cdot}33\times10^{-3}\,M$. These were mixed in the proportions $a:b=3:1$, $1:1$ and $1:3$ and the absorption curves of the five solutions measured. Table II shows found and calculated concentrations of tyrosine and tryptophan expressed in terms of $10^{-3}\,M$. The maximum error in any of the values obtained is +5·0% for tyrosine in mixture $a/b=1$. This compares favourably with chemical estimation of tyrosine and tryptophan in mixtures.

Table II.

Solution		$a/b=3:1$	$a/b=1:1$	$a/b=1:3$	b	a
Tyrosine:	Found	0·59	0·412	0·195	—	0·793
	Calc.	0·589	0·392	0·196	—	0·785
	% error	+0·2	+5·0	−0·5	—	+0·1
Tryptophan:	Found	0·080	0·162	0·248	0·340	—
	Calc.	0·083	0·166	0·248	0·330	—
	% error	+3·6	+2·4	0·0	+0·3	—

Concentrations are given in molarity × 10^{-3}.

II. ESTIMATION OF TYROSINE AND TRYPTOPHAN IN PROTEINS.

Dhéré [1909] suggested that the selective character of the absorption spectrum of proteins was due to the contained aromatic amino-acids. Stenström & Reinhard [1925] showed that a solution containing tyrosine and tryptophan in concentrations calculated from the values of Abderhalden and Kiyotaki for blood serum proteins gave an absorption spectrum very similar to that of serum. They also showed that when a simple solution of tyrosine was made alkaline the absorption curve was shifted towards longer wave-lengths and that the same occurred, though to a lesser extent, on making serum alkaline. Smith [1929] and Coulter *et al.* [1936] have also pointed out the dependence of the selective absorption of proteins on the contained aromatic amino-acids. No quantitative measurements of this dependence are to be found in the literature.

Since it was desired in this laboratory to estimate tyrosine and tryptophan in small quantities (5 mg.) of protein, the spectrophotometric method was investigated as a means to this end. Such application can only be successful (*a*) if the absorption spectra of the aromatic amino-acids are not altered by combination in the protein, and (*b*) if it is possible to select a region of the spectrum where only tyrosine and tryptophan absorb. It is obvious that if a region can be found where only one of them absorbs, then that one may be estimated directly by comparison with a standard solution. Inspection of the absorption curves of amino-acids recorded in the literature [Guthmann *et al.* 1931; Abderhalden *et al.* 1927; 1928; 1929; Ward, 1923] shows that a region can be found ($>280 m\mu$) where tyrosine and tryptophan are the only absorbing amino-acids. Further, there is evidence to show that a chromophoric group is only affected through a system of conjugated double bonds, and that combination of an absorbing molecule with a much larger but transparent molecule is possible without altering the absorption [Holiday, 1930; Arnold & Kistiakowsky, 1932; Gulland *et al.* 1934]. It is therefore feasible that the absorption of tyrosine and tryptophan should not be altered by peptide linking in a protein molecule, since there is no conjugated double bond system linking the chromophoric group (aromatic nucleus) with the site of substitution (—NH_2 or COOH groups). Abderhalden & Haas [1927] have shown that the absorption spectrum of glycyltyrosine is identical with that of tyrosine. If this condition be fulfilled in a protein, then its absorption spectrum will be identical with that of a simple mixture of its contained amino-acids, and

(1) hydrolysis of protein should not lead to any change of absorption;

(2) application of the method of spectrophotometric analysis of mixtures detailed in the previous section should give values of concentrations of tyrosine and tryptophan agreeing with those obtained by chemical determination.

Below are reported results obtained by the chemical and spectrophotometric methods. From considerations discussed in Part I it was found that alkaline solutions of proteins (*N*/10 NaOH) were the most suitable for the measurements. An additional advantage is the fact that most proteins are quite soluble in *N*/10 NaOH.

Experimental.

The source of the proteins. The serum proteins were prepared from horse serum, using the method of Groh & Weltner [1934], in which the serum is diluted 10 to 20 times before precipitation with ammonium sulphate. Besides giving sharp fractionation the samples of euglobulin and pseudoglobulin are remarkably free from pigment, as compared with those obtained from undiluted serum. The euglobulin is that fraction which separates out on dialysing total globulin and which redissolves in 0·9 % saline. The pseudoglobulin is the distilled water-soluble fraction precipitated from serum diluted 10 times with distilled water by 40 % saturation with ammonium sulphate. The albumin is the further fraction obtained by complete saturation with ammonium sulphate. The fractionation of the total globulin was repeated 3 times. All samples were dialysed 7–10 days against distilled water, until the dialysate had been negative 2 days to Nessler reagent. One serum N. IV had been extracted with ether-alcohol according to Hardy & Gardner [1910] as modified by Hewitt [1927]. Except for N. III and N. IV the serum proteins had not been brought to dryness. Concentrations were estimated by N determination by the micro-Kjeldahl method of Parnas & Wagner, using the factor 6·45 to convert to protein. The gliadin was prepared by the method of Dill & Alsberg [1925] and the caseinogen according to the method of Van Slyke & Baker [1918], with the further extraction of the caseinogen with 60% alcohol. This extraction removed an appreciable amount of protein and it is to be noted that the tryptophan content of the extracted protein is considerably lower than before extraction. The two samples of zein were a gift of Dr A. Neuberger. The sample of crystalline insulin examined was a product of Messrs Burroughs and Wellcome. The gelatin was an ash free sample of Eastman Kodak, Ltd.

The concentration of tyrosine and tryptophan in various proteins determined by the spectrophotometric and chemical methods. Absorption curves of proteins were determined on simple watery solutions (when soluble) and on solutions in $N/10$ NaOH. In the case of the serum proteins the concentration was determined by estimation of total N; in other cases weighed quantities of the dried sample were dissolved to give concentrations varying between 0·1 and 0·2%. In order to reduce errors the value of the density measured was always made > 1·0, and in

Table III.

	Tyrosine		Tryptophan		
Protein	Spect.	Chem.	Spect.	Chem.	
Horse serum euglobulin:					
N. E. G. IV	7·7	6·9	2·3	2·1	Dried preparation
IV	7·5	7·2	2·3	2·2	Dried preparation, second separation
V	6·0	—	2·8	—	—
VI	6·8	—	2·9	—	—
Horse serum pseudoglobulin:					
N. P. G. I	6·25	—	3·2	—	—
II	6·4	—	2·8	—	—
III	5·6	6·9 7·2	2·8	2·7	Dried preparation
IV	5·8	5·7	2·6	2·1	Dried preparation
V	5·12	—	2·6	—	—
VI	5·3	—	2·7	—	—
Horse serum albumin:					
1A	(6·3) 7·46	5·5	(0·82) 0·6	0·94	—
2A	(6·45) 8·3	6·23	(0·79) 0·65	0·63	—
Caseinogen:					
A & B	6·95	6·2 6·4 5·7	1·02	1·1 1·0 0·8	—
	7·5	—	1·26	—	—
V. S. & B.	6·7	—	1·04	—	Acc. to Van Slyke & Baker
V. S. & B. E.	6·45	6·55*	0·85	1·4*	Acc. to Van Slyke & Baker but extracted with ether and 60% alcohol
Gelatin	0·46 (0·26)	—	0·05	—	—
Gliadin	(3·4)	3·1 3·4*	(0·65)	0·84 1·14*	—
	4·3	—	0·66	—	—
Zein	(6·3) 10·0	5·9*	—	0·17*	—
Insulin	12·7	12·0†	0·0	—	—

* Given by Folin & Ciocalteau [1927]. † Given by Jensen & Wintersteiner [1932].

most cases was $>1·5$. Plates were matched as described in Part I, and the extinction coefficients (E) of the alkaline protein solutions at 280·0 mμ and 305·0 mμ determined. Substituting these values in the equations previously given, values for molar concentration of tyrosine and tryptophan in the solution examined were obtained from which could be calculated the percentage by weight of the free amino-acids in the protein.

The results are given in Table III, together with the results of chemical analysis. The chemical estimations were made exactly as described by Folin & Ciocalteau [1927] except that 0·1 g. quantities were taken for the analysis. The estimations were not very satisfactory on such small quantities as shown by the divergence of duplicated results.

DISCUSSION OF RESULTS.

Except in the case of N.P.G. III the values for tyrosine are all higher in varying degree than those obtained by chemical determination. Where the discrepancy is large, as in the samples of albumin, gliadin and zein, it can be attributed to the interference of pigment. These samples were all considerably coloured. It is to be noted that this interference does not disturb the tryptophan values to the same extent. The reason for this lies in the form of the equations for the derivation of the concentration of tyrosine and tryptophan:

$$M_{\text{tyrosine}} = 1·0\,E_{305} - 0·092\,E_{280};$$
$$M_{\text{tryptophan}} = 0·21\,E_{280} - 0·288\,E_{305}.$$

If $IE_{305·0}$ and $IE_{280·0}$ be the extinction coefficients at the two wave-lengths due to interfering substances, then the error in molar concentrations (IM) will be:

$$IM_{\text{tyrosine}} = 1·0\,IE_{305} - 0·092\,IE_{280};$$
$$IM_{\text{tryptophan}} = 0·21\,IE_{305} - 0·288\,IE_{305}.$$

In the case of most proteins examined the pigment impurities show "general" absorption gradually increasing with decrease in wave-length, so that IE_{280} is only slightly greater than IE_{305}. The difference is such that $IM_{\text{tryptophan}}$ is very small, whereas IM_{tyrosine} is approximately $0·9\,IE_{305}$. To correct for this it has been found possible to estimate IE_{305} on solutions of proteins soluble in water or dilute acids. In neutral solution or acid solution a mixture of tyrosine and tryptophan shows no absorption beyond 310·0mμ. The absorption of a protein solution at this wave-length has therefore been taken as an estimate of IE_{305}. It has been found that solutions of pigment-free proteins which show little light scattering give a very small value for IE_{305} (limits 0·02–0·06). With the proteins gliadin, zein and serum albumin IE_{305} may be as much as 0·4. Tyrosine values corrected in this manner are given in the table in brackets. These corrected values are not so reliable as those obtained from pigment-free protein solutions.

A final check of the values obtained can be made by comparing the summation curve of tyrosine and tryptophan in the concentrations found with the absorption curve of the protein. Such a summation curve should approximate closely to that of the protein at wave-lengths longer than 275mμ; at shorter wave-lengths than this the protein curve will exceed the summation curve owing to the absorption of other amino-acids. It should also be noted that at no wave-length should the extinction coefficient of the summation curve exceed that of the protein curve by more than the experimental error, i.e. $0·05E$. If this should be found to occur the presence of interfering substances may be suspected, for which it may or may not be possible to introduce a correction. No difficulty was experienced in fitting the curves, except where there was much pigment. A small correction was required in the curves of most serum proteins for the general absorption due to scattering of light. Such solutions generally show visible opalescence in concentrated solution (3–5 %) but none in the concentrations here used (0·1–0·2 %). There is, however, even with the clearest dilute solutions, some scattering of ultraviolet light which must be corrected for.

From the results reported in this paper it is concluded that by the spectrographic method it is possible to arrive at a good estimate of the tyrosine and tryptophan concentrations in a protein. This method has the advantages over the chemical methods of requiring only about 5 mg. of protein, of being much less laborious and subject to small risk of failure in contrast to the case where alkaline hydrolysis must precede estimation.

SUMMARY.

1. The absorption curves of tyrosine and tryptophan have been measured between 260 and 305 mμ in acid, neutral and alkaline solutions.

2. The spectrophotometric method of estimating their concentrations in mixtures of the two is described and the errors of the method discussed.

3. The estimation of tyrosine and tryptophan in proteins by the spectrophotometric method is described.

4. The results are compared with those derived from chemical analysis.

5. The errors of the method and a means of correcting for pigment impurities are discussed.

I wish to express my thanks to Mr W. Weinstein for his technical assistance.

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CCLII. THE USE OF SOME MICRO-ORGANISMS IN SUGAR ANALYSIS.

II. THE QUANTITATIVE DIFFERENTIATION OF FRUCTOSE AND MANNOSE.

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In a previous paper [Harding & Nicholson, 1933] a system of carbohydrate analysis by the use of certain micro-organisms was described. The method made possible the quantitative analysis in dilute solutions of mixtures of glucose, galactose, sucrose, maltose, lactose and fructose and/or mannose. Unfortunately the differentiation of fructose from mannose was not possible by the use of the biological reagents employed at that time.

Kendall [1923] reported *Micrococcus tetragenus* (*Gaffkya tetragena*) as producing acid from glucose, fructose and galactose but not from mannose, lactose or saccharose.

Gaffkya tetragena[1] was found to be suitable for use as an agent for the removal of glucose and fructose. By the use of *G. tetragena*, *P. vulgaris* and *M. krusei* it was possible to differentiate glucose, fructose and mannose.

The use of G. tetragena *in the estimation of fructose and mannose.*

The experimental methods used were the same as those used in the previous paper [Harding & Nicholson, 1933].

Fermentative properties. *G. tetragena* ferments glucose, fructose and galactose with the production of acid but no gas. The fermentation of galactose is very slow, but little acid being produced at the end of 72 hours' fermentation.

Removal power. 1 g. wet weight of the packed organisms removed 1 mg. of glucose or fructose from 10 ml. of solution. No removal of mannose, maltose, sucrose, lactose, arabinose or xylose occurs under the conditions employed. Although the organism ferments galactose, no removal of this sugar by *G. tetragena* takes place during the incubation period used for the removal of glucose and fructose.

Culture. The bacteria are grown on Hartley's broth for 23 hours at 38°, then transferred to the surface of a neutral 2 % glucose-glycerol agar and incubated at 38° for 48 hours. The methods for separating, washing and keeping the cultures of *G. tetragena* are the same as those used for *P. vulgaris* [Harding & Nicholson, 1933].

Estimation of fructose. 2·0 ml. of a 50 % suspension of *G. tetragena* are placed in an aluminium tube and centrifuged in the Lundgren angle centrifuge. The supernatant fluid is poured off and the adherent moisture removed from the tube by filter paper. The solution to be tested is adjusted to *p*H 6·5–7·0 and 10 ml., containing not more than 1·0 mg. of glucose and fructose, are added to the tube, the bacteria are thoroughly mixed with the solution by means of a thin glass rod and the mixture is incubated at 38° for 30 min. The organisms are then removed from the solution, by the methods described for *P. vulgaris* [Harding & Nicholson, 1933]. Ammonium salts if present are removed as $Mg(NH_4)PO_4$ by the addition of KH_2PO_4 and MgO [Harding & Downs, 1933]. The reducing value of the solution before and after the action of *G. tetragena* is determined using the requisite water controls. In the absence of glucose the difference between the total and residual sugar represents fructose. In the presence of glucose the removal figure represents the sum of the glucose and fructose. The glucose is then determined [Harding & Nicholson, 1933] and the difference between the reducing value of the sugars removed by *G. tetragena* and the reducing value of the glucose represents fructose.

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[1] Obtained from the American Type Culture Collection, the John McCormick Institute for Infectious Diseases, Chicago.

Estimation of mannose. Since *G. tetragena* removes glucose and fructose and *M. krusei* removes glucose, fructose and mannose [Harding & Nicholson, 1933], the reducing value of the mannose present in a solution may be obtained by subtracting the reducing value of the residual sugar left after the action of *M. krusei* from that left after the action of *G. tetragena*. The details of the analysis of a known mixture of glucose, fructose and mannose are shown in Table I.

G. tetragena can be used directly on Folin-Wu blood filtrates, in the same manner as outlined above for aqueous solutions of glucose, fructose and mannose.

Table I. *Determination of glucose, fructose and mannose.*

Amounts taken		
5 mg. of glucose in 100 ml. solution: 2 ml. require	0·86 ml.	0·005 N iodine
5 mg. of fructose ,, ,, ,,	0·84 ml.	,,
5 mg. of mannose ,, ,, ,,	0·42 ml.	,,
Total reduction: 2 ml. require	2·12 ml.	,,
	ml. 0·005 N I for 2·0 ml. solution	
Total reduction found	2·10	
Reduction after *P. vulgaris*	1·26	
2·10 − 1·26 = 0·84 = 4·88 mg. glucose removed by *P. vulgaris*		
Reduction after *G. tetragena*	0·43	
1·26 − 0·43 = 0·83 = 4·94 mg. fructose removed by *G. tetragena*		
Reduction after *M. krusei*	0·02	
0·43 − 0·02 = 0·41 = 4·82 mg. mannose removed by *M. krusei*		

The amounts of wet washed organisms used and the time of incubation are those given in Table I [Harding & Nicholson, 1933] and the paragraph "Removal power" in this paper.

* Data from Table I [Harding & Downs, 1933.]

The estimation of fructose and mannose added to urine. Although *G. tetragena* cannot be used on urines cleared with Lloyd's reagent, basic lead acetate or $HgSO_4$-$BaCO_3$ [West *et al.*, 1929] as the action of the organisms produced a definite increase in the reducing value of the filtrates, satisfactory results may be obtained if the urines are cleared with basic lead acetate followed by $HgSO_4$-$BaCO_3$ [West *et al.*, 1929] and the analysis carried out on solutions of the sugars precipitated from such filtrates by a modification of Salkowski's [1879] copper-lime method [Archibald, 1935; Harding *et al.*, 1936].

Table II shows the recovery of added fructose and mannose from fasting human urines treated in the manner just described, the sugars having been added to the urines before the clearing processes were started.

Table II. *The recovery of fructose and mannose added to "fermented" urine specimens.*

Two-hour fasting specimens were treated with *S. marxianus* to remove the "fermentable sugar". A sufficient amount of a solution containing 100 mg./100 ml. of fructose and 200 mg./100 ml. of mannose to give the desired percentage of sugar was then added. This urine + sugar was taken as the urine specimen and dilutions of the final solution were calculated to it.

		Sugar added to original urine, mg./100 ml.		Sugar in final solution, mg./100 ml.			
				Fructose		Mannose	
Urine	Dilution of final solution	Fructose	Mannose	Theoretical	Estimated	Theoretical	Estimated
1	1 : 0·61	2·5	5	4·0	3·9	8·0	7·8
2	1 : 1·66	7·7	15·5	4·7	4·6	9·3	9·6
3	1 : 1·17	2·5	5	2·2	2·1	4·3	4·2
4	1 : 1	5	10	5·0	5·1	10·0	10·4

SUMMARY.

The use of *G. tetragena* affords a ready means of separating fructose and mannose when present in dilute solutions.

Combining its use with that of *P. vulgaris* and *M. krusei* it is possible to analyse mixtures of glucose, fructose and mannose and to recover added fructose and mannose from blood and urine filtrates.

The use of *G. tetragena* necessitates a slight revision in the scheme of analysis for sugar mixtures published by Harding and Nicholson [1933].

A summary of the steps in the revised analysis follows:

A. Estimate glucose, fructose and mannose by *M. krusei.*

B. Estimate galactose in residual fluid from A by *S. marxianus.*

C. Estimate glucose and fructose by *G. tetragena*

$$A - C = \text{mannose}.$$

D. (In absence of galactose) estimate glucose by *P. vulgaris*

$$C - D = \text{fructose}.$$

E. (In presence of galactose) estimate fructose and mannose in residual fluid from D by *M. krusei*

$$A - E = \text{glucose};\ E - \text{mannose} = \text{fructose or } C - \text{glucose} = \text{fructose}.$$

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CCLIII. THE DIGESTION PRODUCTS FORMED BY THE ACTION OF PAPAIN ON EGG ALBUMIN.

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An ultracentrifuge study of the action of papain on egg albumin was made in this laboratory [Svedberg & Eriksson, 1933; 1934], but with the centrifugal forces and methods of measurement then available it was not possible to separate effectively the digestion products obtained. It was therefore thought advisable to use the improved apparatus now in the laboratory to extend these observations. As the ultracentrifuge data still proved insufficient for definite conclusions measurements were also made by other physical methods.

Experimental procedure. The egg albumin was prepared by Sørensen's method [Sørensen, 1917: Sørensen & Høyrup, 1918]. It was crystallized three times, dissolved and dialysed against distilled water, and then electrodialysed. The solution was evaporated *in vacuo* over sulphuric acid and over phosphorus pentoxide, and stored in the dry state in the dark.

The papain used was Merck's Papainotinum 1 : 350. Its activity was tested according to directions given by Willstätter & Grassmann [1924] and found to give results comparable with theirs.

The papain activation with HCN and the treatment of the digestion mixture of papain and egg albumin were carried out in the manner recommended by Willstätter & Grassmann [1924; 1926]. All digestion was done at 40° in acetate buffer of *p*H 5·0. The papain solution was in each case activated at 40° for 2 hours before it was added to the egg albumin. Two different sets of concentrations of digestion mixture and protein solution were used: (*a*) those used by Willstätter & Grassmann for papain activation tests (only slight digestion occurred with these proportions so they were not used in later work), and (*b*) those concentrations used by Willstätter & Grassmann in egg albumin digestion. These concentrations were as follows:

(*a*)	(*b*)
4·8 % egg albumin	4·0 % egg albumin
0·12 % papain	0·3 % papain
0·24 % hydrogen cyanide	0·1 % hydrogen cyanide
0·04 *N* acetate buffer	0·02 *N* acetate buffer

To prevent continued digestion solutions were kept at 4° after removal from the digestion oven. Further, the HCN was removed by reducing the air pressure above the solution; this was done over a water-bath to minimize solution evaporation. All dialysis took place at 4°.

I. *Ultracentrifuge measurements.* Descriptions of the theory and technique of ultracentrifuge experiments and details of the methods of calculating the results have already been given [Svedberg, 1933; McFarlane, 1935]. The absorption method of measurement was only used with undigested solutions and as a means of testing whether digestion had occurred. It requires far less time for the calculation of results than the refractive index method, but the latter is much more suitable for the separation of components of a mixture.

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[1] Holder of Royal Society of Canada Fellowship.

The solutions used for centrifuge analysis contained 0·02 N acetate buffer pH 5·0, 0·2N sodium chloride, and approximately 1 % protein. The sodium chloride was added to suppress the Donnan effect produced when charged particles move through a liquid under the action of a centrifugal field [Tiselius, 1932].

It was found that the papain caused the egg albumin to break up into two fractions. There was no observable change in the nature of these two fractions with length of time of digestion, only in their proportions. The concentration of the lighter fraction increased at the expense of the heavier one as digestion proceeded until the latter had completely disappeared.

The sedimentation diagrams of the heavier fraction showed no asymmetry indicative of heterogeneity. Its average sedimentation constant (S_{20})[1] was 3·30, but the deviations of individual values were a little larger than would have been expected from a consideration of the experimental errors involved. This suggests that the fraction was a mixture of materials with nearly equal sedimentation constants, the composition differing slightly under different conditions. The accepted value for the sedimentation constant of undigested egg albumin is 3·55. The results are shown in Table I.

Table I.

Method of measurement (absorption or ref. index)	Digestion time hours	$S_{20} \times 10^{13}$ sec.	Remarks
A	0	3·59	
RI	0	3·49	Average 3·54
		(Heavier component.)	Digestion proportions (a) (Only slight digestion occurred with these proportions)
RI	45	3·29	Same solution
A	45	3·22	
A	93	3·25	—
RI	117	3·20	Same solution
A	117	3·27	
A	120	3·20	—
			Digestion proportions (b)
RI	0·5	3·40	—
RI	5	3·36	—
RI	19·5	3·31	Same solution
A	19·5	3·14	
RI	24	3·14	—
RI	24	3·49? S_1	—
RI	24	3·58? S_2	—
RI	43	3·32 S_1	—
RI	43	3·41 S_2	$S_2 = S_1$ with the light component removed by dialysis
RI	45	3·33 S_1	—
RI	45	3·38 S_2	—
RI	48	3·15	—

Even centrifugal forces 410 × 10^3 times gravity were not sufficient to cause the peak of the sedimentation curve of the lighter fraction to break away from the meniscus, i.e. the material was so light that the sedimentation diagrams obtained were those of the early stages of sedimentation equilibrium rather than of sedimentation velocity experiments. It was therefore not possible to compute a sedimentation constant for this fraction, but calculation showed that it must be less than 0·2. The lighter fraction passed through a cellophane membrane so that it was possible to separate the two fractions of the digestion mixture by dialysis. This effect is illustrated in Figs. 1 and 2. Fig. 1 shows a sedimentation diagram for a solution digested for 45 hours; Fig. 2 shows a corresponding sedimentation diagram for the same solution after the light component had been dialysed away through a cellophane membrane.

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[1] S_{20}—sedimentation velocity in pure water at 20° under unit force. It is always expressed in units of 10^{-13} seconds.

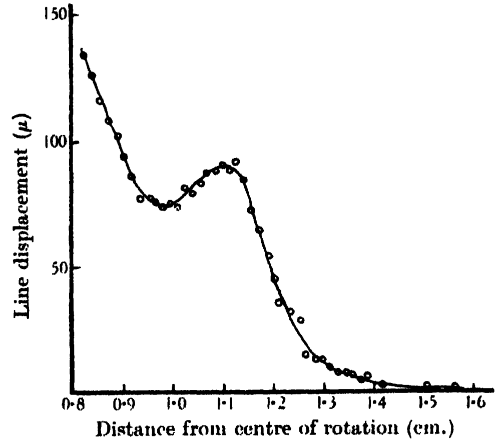

Fig. 1.

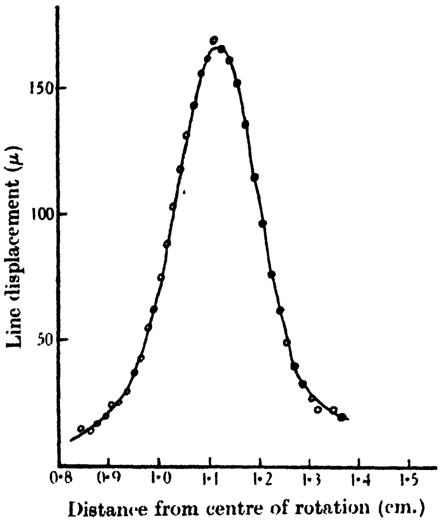

Fig. 2.

The light component gave no precipitate with salicylsulphonic acid, a slight precipitate with lead acetate, and a dense one with phosphotungstic acid.

Previous experiments had shown that keeping the egg albumin at 40° had no effect on its behaviour in the ultracentrifuge, nor had treating it with unactivated papain.

Except when the time of digestion was less than 5 hours, a reference scale made with a solution containing no papain gave better (i.e. more horizontal) base lines in the sedimentation diagrams than a reference scale made with a solution containing papain in the concentration present in the digestion mixture. This suggests the possibility that the material contained in the papain preparation was combined with the egg albumin molecules. No evidence could be found of heavy albumin-papain particles but their number would be so small and their weight such as to make separation from the principal component in the sedimentation diagrams very difficult. From reference scales made with and without papain it was possible to deduce that the sedimentation constant of the papain preparation was about 2·8.

II. *Refractive index measurements.* The purpose of these measurements was to study quantitatively the time variation of the relative amounts of the two fractions of digestion products. Seven samples of the same egg albumin-papain mixture (digestion proportions (*b*)) were placed in an oven at 40° and removed after various lengths of time of digestion. The two fractions in each sample were separated by dialysis through cellophane membranes. The refractive index differences between each of these solutions and a standard buffer solution were measured with a Pulfrich refractometer ($\lambda = 546\ m\mu$). Assuming these refractive index differences to be proportional to the concentrations the total amount of material in each solution was calculated. This assumption is not strictly justified [v. Hand, 1935]. The increase during digestion in the apparent total amount of material present is probably attributable to variations in refractive increment. The results have been plotted in Fig. 3.

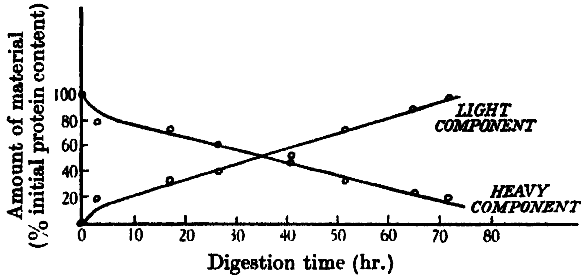

Fig. 3.

As digestion proceeded the proportion of the lighter fraction increased at the expense of the heavier one. The process was a linear function of the time of digestion except for a short initial period, not greater than 5 hours, during which the action was somewhat more rapid.

III. *Diffusion measurements.* The diffusion apparatus was that developed by Tiselius & Gross [1934] and by Lamm & Polson [1936]. The method of measurement was of the refractive index type similar to that used to measure concentration gradients in the ultracentrifuge. Series of experiments were made using solutions digested for various lengths of time and carefully separated into two fractions by dialysis through cellophane membranes. All experiments were made at 20° in acetate buffer 0·02 N pH 5·0. Sodium chloride (0·2 N in most cases) was used to depress the Donnan effect. The protein concentration was approximately 1 %.

The homogeneity of the diffusing materials was tested by comparing concentration distribution curves with the corresponding ideal diffusion curves calculated by means of the formulae developed by Pearson [1894]. The diffusion constants (D') were calculated from the ideal curves using equation (1)

$$D' = \frac{\mu^2}{2t}\left(\frac{l-b}{l}\right)^2 \qquad \text{......(1).}$$

μ = half the distance between the two inflexion points. The position of the inflexion points was obtained by dividing the maximum height of the curve by $\sqrt{e}$.

t = the time since diffusion started.

l = the optical distance from the scale to the camera objective.

b = the optical distance from the scale to the centre of the diffusion tube.

These values were corrected for the viscosity of the salts present in the solution by means of equation (2)

$$D = D'\frac{n_s}{n_w} \qquad \text{......(2).}$$

n_s = the viscosity of the solvent at 20°.

n_w = the viscosity of distilled water at 20°.

For comparison with the values for the light component, the diffusion constants of two amino-acids and one tripeptide were measured. For these experiments no buffer solutions were used, but the materials were dissolved directly in freshly boiled distilled water in order to be as nearly as possible at their isoelectric points. As the magnitude of the charge on the particles was not known, a high sodium chloride concentration (0·5 *N*) was used to suppress Donnan effects.

Table II.

Material	Time of diffusion hours	D'	$D \times 10^7$ cm.^{2}/sec.
Light component:			
36 hours' digestion	1	51·3	
	4	50·4	52·[illegible]
45 hours' digestion	3	44·7	
	5	47·1	46·6
70·5 hours' digestion	2	57·2	
	3	55·3	56·5
70·5 hours' digestion	2	54·2	56·8
Heavy component:			
27 hours' digestion	13	7·52	
	21	7·71	7·80
36 hours' digestion	20	8·24	
	24·5	8·15	8·20
44·5 hours' digestion	9	8·38	
	19	8·42	8·40
Glycine (mol. wt. 75)	1·0	91·3	
	1·5	85·8	
	1·75	84·6	
	2·5	85·7	90·8
l-Tryptophan (mol. wt. 204)	1·5	53·8	
	2·0	55·7	
	2·5	55·8	57·3
dl-Leucyl-glycyl-glycine (mol. wt. 245)	1·5	43·9	
	2·5	43·6	46·1

The diffusion experiment results are given in Table II. Both light and heavy components were found to be heterogeneous, i.e. there was a marked difference between the experimental and the ideal diffusion curves. The heterogeneity was definitely larger than could be accounted for by incomplete separation of the two components in dialysis. The diffusion constants listed have been calculated from the ideal diffusion curves most nearly corresponding to the experimental data and must therefore be regarded as average values for the various substances present. There was some indication that in each case the average diffusion constant increased slightly with the time of digestion. The glycine, *l*-tryptophan, and *dl*-leucyl-glycyl-glycine gave ideal diffusion curves. From a consideration of these diffusion constants it is evident that the lighter fraction of the digestion mixture must have contained some substances having diffusion constants of the same order of magnitude as the amino-acids, and probably some of the order of magnitude of the lower polypeptides as well. The average diffusion constant of the heavier component (8·27 cm.^{2}/sec.) was a little higher than that of undigested egg albumin (7·76 cm.^{2}/sec.).

IV. *Cataphoresis measurements.* These measurements were made with the moving boundary apparatus developed by Tiselius [Tiselius, 1930; Pedersen, 1933]. Both absorption and refractive index methods of measuring the concentration gradients were available. The former method was used with the light component. Samples were tested after 27 hours' and after 45 hours' digestion.

They were found to be so heterogeneous that calculations of mobility were not possible. At *p*H 5·0 the average mobilities were almost zero, but they contained components having anodic and some having cathodic motion.

The refractive index method was used to study the heavy fraction. This necessitated using 2 % solutions. As mobilities of undigested egg albumin solutions of such high concentration had not been measured previously by this method, these were investigated first. Solutions were used which had been kept at 40° in buffer but without papain, to test whether the heat alone had any effect on the mobility of the egg albumin. The concentration distribution curves for this solution showed no trace of asymmetry. The mobility calculated from these curves was exactly that obtained for this *p*H by Tiselius [1930] using dilute solutions [v. however Smith, 1935; 1936]. At *p*H 5·01 the motion was anodic $4{\cdot}5 \times 10^{-5}$ cm./sec./volt/cm.

The heavy fraction gave concentration distribution curves which were very asymmetrical, showing marked heterogeneity. Consideration of these curves shows that the heavy fraction was made up of several components. The principal component had an anodic mobility of $1{\cdot}4 \times 10^{-5}$ at *p*H 5·0 (digestion time 36 hours), and $1{\cdot}9 \times 10^{-5}$ at *p*H 5·4 (digestion time 24 hours). The other components all had mobilities less than these so that no material remained having a

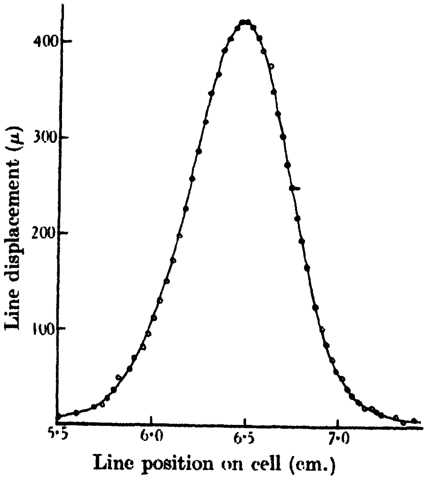

Fig. 4.

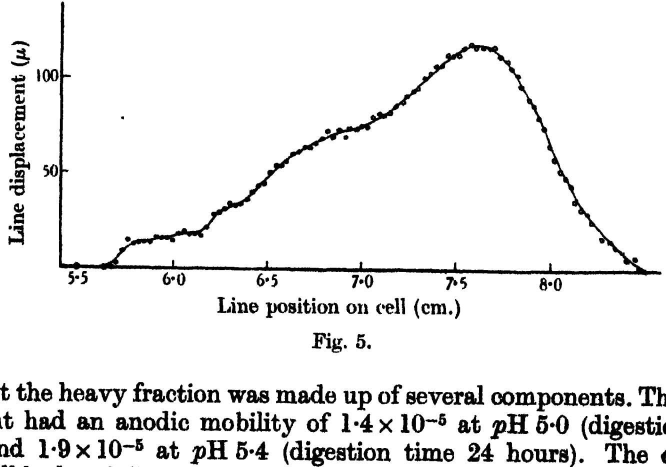

Fig. 5.

mobility equal to that of the undigested egg albumin. A typical concentration distribution diagram of the undigested egg albumin is shown in Fig. 4, and a typical curve for the heterogeneous heavy fraction is shown in Fig. 5.

V. *Light absorption measurements.* The light absorption of the undigested egg albumin and of the light and heavy fractions of the digestion mixture were measured by means of a Judd-Lewis spectrophotometer. Tests were also made of the intensity of light λ 270 $m\mu$ (obtained by means of a mercury lamp and chlorine and bromine filters) transmitted by a quartz cell containing the liquids.

The results cannot be regarded as very definite as concentrations were measured by means of refractive index measurements, and no account was taken of possible variations in refractive increment. The light fraction of the digestion mixture appeared to have a distinctly higher and the heavy fraction a somewhat lower extinction coefficient than the undigested egg albumin. There was no change in the positions of the wave-lengths of maximum or minimum absorption. The absorption of light by egg albumin in the region 253–290 $m\mu$ is almost entirely due to the aromatic amino-acids phenylalanine, tyrosine, and tryptophan [v. Coulter *et al.* 1936]. Thus an increase in the extinction coefficient of the lighter component would mean that the relative concentration of one or all of these substances had increased.

DISCUSSION.

It seems probable that the digestion of egg albumin by papain takes place in at least two stages: (1) a change in all of the molecules, probably a loosening of bonds within the egg albumin molecule and (2) a gradual splitting off of small pieces from these modified molecules.

Evidence for the first stage is furnished by cataphoresis measurements: partially digested solutions contain no component having a mobility equal to that of undigested egg albumin. Further, the sedimentation constant of the material is decreased even before any appreciable amount of decomposition products has been formed. This is in agreement with the previous investigation of this problem [Svedberg & Eriksson, 1934] in which a fraction was isolated from the digestion mixture of unchanged molecular weight but having a decreased sedimentation constant.

All the experiments show that very small molecules are produced during the second stage of digestion. Ultracentrifuge and diffusion experiments show that these must be of the order of magnitude of amino-acids and lower polypeptides. If we neglect changes in partial specific volume—the changes may be quite large [v. Cohn *et al.* 1934]—we may get an approximate idea of the average molecular weights of the components formed during digestion using the relationship

$$\underset{\text{(Fraction)}}{\text{Mol. wt.}} = \underset{\text{(Egg albumin)}}{\text{Mol. wt.}} \times \frac{\underset{\text{(Fraction)}}{\text{Sed. const.}}}{\underset{\text{(Egg albumin)}}{\text{Sed. const.}}} \times \frac{\underset{\text{(Egg albumin)}}{\text{Diffn. const.}}}{\underset{\text{(Fraction)}}{\text{Diffn. const.}}}.$$

This gives a molecular weight of 38×10^3 for the heavy fraction and about 4×10^2 for the light fraction. The molecular weight of undigested egg albumin is 42,200. This suggests that certain definite parts are split off from the molecule rather than that some molecules are simply broken up into many small pieces. This is in agreement with the fact that the light component seemed to contain a higher percentage of the aromatic amino-acids than undigested solutions.

Refractive index measurements show that except for a short initial period the process of digestion is a linear function of the time (see Fig. 3). This corresponds to a monomolecular reaction taking place always at saturation. The change of slope of the initial part of the curve points to the presence of some other factor during this part of the digestion process.

SUMMARY.

Under the action of papain the egg albumin molecule is split up into two groups of substances. Both fractions are heterogeneous. The light fraction consists of particles of the order of magnitude of the amino-acids and the lower polypeptides. The heavy fraction has an average sedimentation constant of 3·30 and an average diffusion constant of 8·27 cm.^{2}/sec. The heavy fraction contains no unchanged egg albumin. It is suggested that the first stage in digestion consists in a loosening of bonds within the molecule and that subsequently small parts are split off from these modified molecules. Ultracentrifuge, refractive index, diffusion, cataphoresis and light absorption measurements have been used in arriving at these conclusions.

The author wishes to express her sincere thanks to Prof. Svedberg for suggesting the problem, for his interest throughout the investigation, and for the privilege of working in his laboratory. She is greatly indebted to Mrs I.-B. Eriksson-Quensel for advice and criticism throughout the research. She also desires to thank Dr K. O. Pedersen, Mr A. G. Polson and Mr K. Andersson for their assistance with cataphoresis, diffusion and absorption measurements respectively.

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CCLIV. A NOTE ON THE RELATIVE DISTRIBUTION OF PHOSPHORUS AND PHOSPHATASE ACTIVITY IN THE FLORAL PARTS OF *NICOTIANA AFFINIS*, *PETUNIA*, *SALPIGLOSSIS* AND *GLADIOLUS*.

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THE activity of phosphatase in the bean, potato, radish and wheat at different stages of their life histories was reported in a previous paper [Ignatieff & Wasteneys, 1936]. The small amounts of material available however made the investigation of the distribution of the enzyme in the various parts of the flower impossible. Such data are needed to complete our knowledge of the distribution of the activity of phosphatase in the higher plants.

EXPERIMENTAL.

The method used for the estimation of the enzymic activity was the same as that described in the previous paper.

The first determinations were made on *Nicotiana affinis*, *Salpiglossis* and *Petunia* (Table I). The flowers were divided into petals, sepals and sex organs, and it is clear that in *Salpiglossis* and *Nicotiana affinis* the sex organs have a higher phosphatase activity than the other floral parts or the leaves. In *Petunia* there was no greater phosphatase activity in the sex organs than in the leaves and sepals. If the relatively high phosphatase activity in the sex organs is related to their functions, such a relationship should exist in all higher flowering plants. On the other hand it should be borne in mind that there is a great variation in the relative size of the different parts of the sex organs of the different species of flowering plants. It is possible also that there are differences in the concentrations of phosphatase present in the various sex organs, and therefore the activity of the enzyme in these organs when tested collectively will depend not only on the actual concentration of phosphatase present but also on their relative size. More accurate information on the distribution of phosphatase

Table I. *Phosphatase activity in the leaves and in the flowers of* Nicotiana affinis, Petunia *and* Salpiglossis.

	mg. of inorganic phosphorus released from 2% sodium β-glycerophosphate solution by 1 g. of dry tissue in 1 min.		
Parts of the plant	*N. affinis*	*Petunia*	*Salpiglossis*
Leaves	0·95	2·93	0·47
Spathes	0·65	2·91	0·51
Petals	0·61	1·71	1·14
Sex organs	1·39	2·97	4·52

in the floral parts of the plants is obtained, if the activity of the enzyme determined in anthers, filaments, styles and ovaries separately. *Gladiolus* was selected for such determinations because it possesses large sex organs.

Three different groups of flowering spikes, designated respectively A, B and C, were obtained. The flowers were removed from their spikes and were separated into their component parts as soon as possible, but as this process could not be completed in 1 day the material was kept overnight in a cold room at about 38° F. At this temperature the metabolic activity and the transpiration in the plants are very low, so that no significant changes could have occurred. The flowers were divided into petals, spathes, filaments, anthers, styles and ovaries. Samples of the leaves were also taken. After the flowers had been removed from the spikes, the latter were placed in water to allow the rest of the flower buds to blossom. In this manner two or three crops of flowers were collected from the same group of spikes, each crop being designated by a number placed after the letter which established the identity of the group.

The results of the experiments on *Gladiolus* are reported in Table II. From these results it is clear that the anthers show the greatest and the leaves the lowest enzymic activity. The leaves, however, towards the end of the life cycle of the plant contain large quantities of less vitalized fibrous material which may account for their low phosphatase activity. The activity was almost as low in the spathes and, indeed, there is no significant difference in the activities of the phosphatase in these two parts. The enzymic activity of the petals of the first crop of flowers removed from the spikes was also low, being equal to that of the spathes, but there was an increased activity in the petals of the flowers which

Table II. *Phosphatase activity in the leaves, spathes, petals and individual sex organs of* Gladiolus.

mg. of inorganic phosphorus released from 2% sodium β-glycerophosphate solution by 1 g. of dry tissue in 1 min.

	Group A			Group B	Group C	
Parts of the plant	Crop 1	Crop 2	Crop 3		Crop 1	Crop 2
Leaves	—	0·13	0·18	0·22	0·13	—
Spathes	0·15	0·17	0·18	0·24	0·25	0·23
Petals, white	0·20	—	—	0·30	0·44	—
Petals, coloured	0·16	0·35	0·65	0·24	0·36	0·59
Ovary	0·43	0·51	—	0·41	0·48	0·46
Style and stigma	0·28	0·60	0·60	0·49	0·57	0·71
Filaments	0·48	0·59	0·54	0·67	0·40	0·42
Anthers	1·19	1·48	1·41	1·15	1·19	1·18

Table III. *Phosphorus distribution in the leaves, spathes, petals and individual sex organs of* Gladiolus.

Total phosphorus in mg. per g. of dry matter

Parts of the plant	Group B	Group C_1
Leaves	1·68	1·79
Spathes	1·97	2·07
Petals	2·43	2·45
Ovary	4·65	—
Style and stigma	3·00	3·42
Filaments	3·63	3·74
Anthers	6·70	6·99

bloomed while the spikes were standing in water. On several occasions phosphatase activities in white and coloured petals were separately determined but no significant differences were found. The enzymic activity in the filaments, styles and ovaries was, in general, higher than in the petals.

Total phosphorus determinations were made on different parts of the flowers of groups B and C_1, according to Cockefair's method [Cockefair, 1931]. The results are presented in Table III and show that the phosphorus concentration in the sex organs is higher than in the petals, spathes or leaves and is greater in the anthers than in any of the other sex organs.

The variation in size of the floral parts is clearly shown by the results of total dry weight determinations shown in Table IV.

Table IV. *Dry weight* (*g.*) *of different parts of* Gladiolus *flower.*

Spathes	0·058
Corolla	0·139
Ovary	0·013
Style and stigma	0·005
Filaments	0·006
Anthers	0·010

Discussion.

In the previous paper [Ignatieff & Wasteneys, 1936] it was stated that the phosphatase activity in the flowers was approximately the same as in the leaves at maturity of the plant. Whilst the results here reported appear to be at variance with this, the explanation for the difference is provided by the determinations of enzymic activity in the floral components of *Gladiolus* (Table II), which show that whilst the phosphatase activity is greater in the sex organs as a whole than in other parts of the flowers or in the leaves, it is only the anthers which have markedly higher phosphatase activity, and these, as Table IV shows, constitute only one-twentieth of the total dry weight of the flowers. Moreover, in *Gladiolus* and in *Salpiglossis* the leaves at the time of flowering contain, as compared with those of the other plants whose phosphatase activity is reported, a high proportion of inert fibre which explains the more marked difference between flowers and leaves in these two plants.

The very high phosphatase activity in the anthers of the *Gladiolus* is of special interest in view of the very high concentration of total phosphorus in these organs.

At present it is unwise to generalize but since in *Gladiolus* the sex organs have greater phosphatase activity, as well as a greater phosphorus concentration, than the spathes and petals, it may perhaps be suggested that in the flower the sex organs and especially the anthers are the site of the most active phosphorus metabolism. This hypothesis finds further support in Cockefair's work [Cockefair, 1931].

Unfortunately it was impossible to study the phosphatase activity throughout the life histories of the flowering plants investigated, and therefore no comparisons can be made between the enzymic activity of the floral parts and that of the other portions of the plant at an active stage of growth. Such a study would be of great interest. It was shown in the previous paper [Ignatieff & Wasteneys, 1936] that young leaves have a very high concentration of the enzyme.

SUMMARY.

Phosphatase activity of the floral parts of *Nicotiana affinis*, *Salpiglossis*, *Petunia* and *Gladiolus* was determined. It was found to be higher in the sex organs as a whole than in other floral parts. In the case of *Gladiolus* it is the anthers which especially are responsible for this higher activity.

Total phosphorus estimations which were made on the floral parts of *Gladiolus* showed the phosphorus concentration of sex organs to be higher than that of spathes and petals, and again it is the anthers which have the highest content of phosphorus.

The author wishes to express his thanks to Prof. Hardolph Wasteneys for his advice and interest in this work, and also to Messrs Donald Robertson and Lionel Ignatieff for their assistance in handling the flowers.

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CCLV. FASTING AND REALIMENTATION IN THE RUMINANT.

I. THE EFFECT OF FOOD AND FASTING ON CERTAIN BLOOD CONSTITUENTS.

II. CALCIUM AND PHOSPHORUS METABOLISM DURING FASTING, AND DURING REALIMENTATION FOLLOWED BY FASTING.

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I. THE EFFECT OF FOOD AND FASTING ON CERTAIN BLOOD CONSTITUENTS.

In the course of a study of the precursors of milk fat it became necessary to have detailed information regarding not only the nature and distribution of blood lipins in the cow but also their variation under experimental conditions. The existing information concerning blood lipoids, although largely derived from micro-determinations and therefore to be accepted with reserve, permits certain general conclusions so far as carnivora and non-ruminant herbivora are concerned. It is recognized [Terroine, 1914, 1, 2; 1919] that wide variations in blood lipoids exist between different species and between individual members of the same species although for a particular animal a certain degree of constancy is characteristic.

Of the factors which influence the various constituents of blood, one of the most important is diet; the present work deals with this factor in so far as it affects ruminants.

Feeding with high fat diets has been found to produce an increase in total lipoids in dogs and rabbits [Bloor, 1932]. A similar lipaemia has been observed after a single fatty meal in dogs [Reicher, 1911; Terroine, 1914, 1, 2; 1919; Bloor, 1915, 1916, 1921; Bang 1918 and others], but not in the rabbit [Sakai, 1914; Bloor, 1921; Iwatsuru, 1925], and not invariably in man [Mann & Gildea, 1932].

The results of Maynard & McCay [1929] and of Porcher & Maynard [1930] indicate that alimentary lipaemia is inconspicuous or non-existent in cows, probably owing to the slowness of digestion and absorption in these and other ruminants. It is evident that fasting in such animals must be prolonged in order both to make clear its effects on blood lipoids and to reduce the latter to their basal level.

In addition to a study of the blood lipoids, it is important that the normal values for certain other blood constituents, such as iron, sugar, amino-N and P should be established and that their variations in response to stimuli should be determined. It is true that the role of P has been widely investigated, but as yet little has been recorded as to the distribution in blood of the various forms of this element under varying physiological conditions.

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[1] Agricultural Research Scholar.

With these considerations in view the investigations recorded in the present communication were undertaken.

EXPERIMENTAL.

Methods.

Throughout this work a portion of each sample of blood was used to obtain an alcohol-ether extract in the usual way (1 part blood in 20 parts of 3 : 1 alcohol-ether mixture). Lipin-P was estimated colorimetrically by King's [1932] modification of the Fiske and Subbarow method, cholesterol by digitonin, and total fatty acids by a slightly modified form of the Stoddart and Drury technique. Blood sugar was determined by the improved form of the Hagedorn and Jensen method described by Fujita & Iwatake [1931], amino-N by the colorimetric procedure of Folin [1922], chloride by the Whitehorn [1921] thiocyanate method, and iron by the technique of Blackwood & Stirling [1932]. Calcium was titrated as oxalate using the precipitation technique of McCrudden [1909–10; 1911–12] and the washing methods of Clark & Collip [1925].

Exp. 1. A cow which had been receiving a normal diet was starved for 24 hours and then given a test meal of linseed oil 1, oats 5, straw 7 and flaked maize 1 lb. The fat and P intakes were estimated to be 340 and 8·92 g. respectively, 340 g. fat being equivalent to 0·85 g./kg. body weight. Twelve blood samples were taken alternately from each mammary vein, this procedure being adopted to minimize the inflammation caused by taking a large number of samples over a short period from the same vein. The results recorded in Table I appear to justify this alternate sampling.

Table I. *Two-hourly sampling after a meal. Distribution of various constituents (mg./100 ml. blood).*

Hours before or after meal	Lipin-P	Total fatty acids	Free sterol	Sterol as ester	Iron	Sugar	Amino-acid N	In-organic P	Organic acid-soluble P
Before 3	9·20	225	76·1	64·3	40·3	50·3	8·34	5·55	1·91
1	9·30	227	77·8	55·4	38·9	44·5	7·98	5·60	2·04
After 1	9·49	—	78·7	61·7	39·3	43·6	7·98	5·06	2·38
3	—	—	77·8	—	40·3	37·8	9·37	5·25	2·44
5	9·19	231	78·1	53·1	38·1	44·5	7·24	5·44	—
7	9·60	225	81·6	51·0	42·8	44·2	7·92	4·94	2·88
9	9·71	225	82·6	60·0	40·3	55·9	9·17	5·26	2·53
11	9·42	225	81·3	49·9	41·0	53·3	8·55	4·92	2·60
13	9·67	225	78·4	55·6	39·7	54·4	8·41	5·27	2·54
15	9·51	216	79·7	50·7	40·1	53·3	9·31	4·83	2·58
17	9·56	216	80·0	48·8	38·1	57·0	7·33	4·86	2·76
19	9·40	212	78·4	46·0	37·9	52·1	8·85	4·56	2·73
Mean values	9·46	223	79·2	54·4	39·7	49·2	8·37	5·13	2·49
Standard deviations	0·18	6·7	2·14	3·40	2·61	6·3	1·13	0·28	0·28

The interval between the previous meal and the test meal was 24 hours.

In case a basal level, differing from the normal absorption level, if such exists, might not have been attained in the 24-hour period of fasting previous to the test meal, Exp. 2 was devised.

Exp. 2. A cow which had previously been on a normal diet was given its final ration at 10 p.m. and was then deprived of food for 5½ days, being allowed water *ad lib.* Rumination ceased on the 3rd day and about the same time dilatation of the pupils was noticed, but no other ill effects. Care was necessary in realimentation and so immediately after the last fasting sample had been taken, 6 lb. hay were given to induce rumination and promote normal digestion. 4 hours later the following meal was given: linseed oil 2, oats 3, straw 7, flaked maize 1 lb. The fat and P intakes were 529 and 13·0 g. respectively, 529 g. fat being equivalent to 1·32 g./kg. body weight. For the next 3 days 12 lb. hay were given daily, and no difficulty was experienced in realimentation. The times of sampling and the results will be clear from Table II.

Table II. *Blood constituents during 5½ days' fast and after a meal (mg./100 ml. blood).*

Time after last meal (days)	Lipin-P	Total fatty acids	Free sterol	Sterol as ester	Iron	Sugar	Amino-acid N	In-organic P	Organic acid-soluble P
½	8·68	159	79·8	45·8	34·5	46·5	6·65	3·67	2·45
1½	8·36	161	78·8	48·4	38·3	36·9	4·53	3·76	2·20
2½	7·56	160	75·8	43·4	39·7	42·8	6·62	4·33	2·73
3½	7·89	160	76·2	51·0	35·2	35·9	4·97	5·46	2·64
4½	7·57	165	76·4	49·6	36·2	49·3	5·36	5·87	2·91
5½	7·57	149	75·4	45·4	40·0	51·9	6·09	6·78	2·86
Time after experimental meal (hours)*:									
8	7·60	156	75·6	51·2	37·7	51·7	7·53	7·39	3·14
20	7·53	135	74·6	43·0	38·5	44·4	9·46	6·93	3·64
32	7·92	135	73·6	51·2	35·5	43·8	9·94	7·28	3·51
44	7·52	137	69·2	49·6	32·6	43·3	8·82	7·46	2·38
68	7·40	132	70·6	58·2	33·9	42·2	9·20	5·95	2·66
8 days	7·54	130	67·9	52·9	31·9	44·7	6·85	5·72	2·80

* The test meal was given 4 hours after the previous sample had been taken. The next sample was obtained 8 hours after the administration of the meal.

Table III. *Constituents of blood during a 7½-day fast recorded (in brief), and during a 10½-day fast (recorded in detail) (mg./100 ml. blood).*

Time before or after meal	Lipin-P	Total fatty acids	Free sterol	Sterol as ester	Iron	Sugar	Amino-acid N	Ca	Cl	In-organic P	Organic acid-soluble P
Exp. III:											
12 hours before	10·34	204	89·1	51·4	48·5	47·0	7·66	5·73	—	1·17	2·91
12 hours after	10·21	191	92·6	54·6	47·0	38·6	7·08	6·01	—	3·63	2·65
2½ days after	9·74	192	91·1	52·1	47·0	45·1	8·36	6·15	—	3·60	3·12
4½ days after	9·86	208	96·6	46·0	49·3	49·7	8·41	6·59	—	4·19	3·28
7½ days after	9·54	179	94·4	36·1	49·3	68·6	6·54	5·85	—	4·01	3·56
Meal given immediately											
24 hours later	9·81	211	95·8	37·9	56·5	68·6	5·99	5·81	—	4·23	3·91
48 hours later	10·20	—	98·1	34·7	51·9	70·5	—	5·93	—	3·82	3·61
Exp. IV:											
12 hours before	9·44	198	92·4	57·9	41·7	52·2	6·07	6·70	268	3·86	3·37
12 hours after	10·04	203	95·9	39·0	37·1	71·9	7·05	7·33	267	3·94	2·58
1½ days after	10·37	214	96·3	37·4	43·5	65·5	7·12	7·21	261	4·92	2·35
2½ days after	10·27	—	98·5	54·7	37·1	65·1	5·10	6·43	266	4·72	3·27
3½ days after	10·06	224	95·7	58·0	43·9	44·9	—	6·75	279	4·95	3·19
4½ days after	9·56	186	91·1	59·2	—	50·5	5·81	6·63	276	5·08	3·12
5½ days after	9·54	201	87·6	48·1	41·4	52·4	6·23	6·40	280	4·86	2·99
6½ days after	9·39	204	87·5	46·3	—	51·9	6·12	6·75	276	4·61	2·55
7½ days after	8·70	207	85·0	53·9	43·4	47·3	6·88	6·32	265	4·53	2·66
8½ days after	8·25	207	91·4	37·4	—	49·9	6·84	6·63	271	4·44	2·63
9½ days after	8·89	213	89·3	46·2	—	49·1	5·10	5·89	267	3·95	2·96
10½ days after	8·36	176	89·1	40·6	43·4	46·5	6·88	6·90	267	3·76	2·86
Meal given immediately											
24 hours later	8·33	178	84·7	39·6	38·3	38·0	8·26	6·55	268	3·74	3·03

Previous to the fast the animals were on a low protein diet for a week.

Exps. 3 and 4. Two non-lactating cows were subjected to periods of 7½- and 10½-day fasts respectively, and in view of the paucity of starvation experiments on ruminants, metabolism trials were run and will be described later. Blood samples were taken from alternate subcutaneous abdominal veins at daily intervals. The animals finished the experimental periods in good condition. The results are set out in Table III.

DISCUSSION.

In Table I it is clear that the ranges of variation of all the constituents studied are extremely small, the contrast between the mechanism of digestion and absorption in the ruminant and that in one-stomached animals being most obvious. The standard deviations are recorded and these may be used in subsequent experiments to test the significance of any apparent changes over a similar period.

In view of the results of Channon & Collinson [1929, 1, 2] indicating that the glyceride fraction of blood fat may be very small or even absent, the distribution of the lipoids was calculated, using the factors generally adopted. On this basis the mean values recorded for the fatty acids in Table I suggested the following distribution: as phosphatides 172·6, as steryl esters 39·6, and as glycerides 10·5 mg. fatty acids per 100 ml. blood. That the figure for the glycerides is exceedingly low or zero can readily be confirmed in the same way from the other three experiments. These findings are therefore in agreement with those of Channon & Collinson, but it must be realized that the factors used are those adopted mainly for liver phosphatide and may not be strictly applicable to blood, which contains a higher proportion of sphingomyelin. In fact Lintzel [1934] has suggested the factor 16·5 to 16·8 for converting blood lipin P to phosphatide instead of 18·5 as used for liver. Lintzel also finds an appreciable difference between the total alcohol-ether-soluble P and the acetone-precipitated P. As the identity and magnitude of the glyceride fraction of blood deserve further study in view of the possible importance of these compounds in the secretion of milk fat, the whole question is receiving further attention.

During the much longer fasting period of Exp. 2, there appeared to be a significant decrease in lipin P, realimentation restoring the initial level. There was no significant change, however, in the total fatty acid and sterol fractions which did not show any tendency to reach a basal level markedly dissimilar from the normal.

In Exps. 3 and 4, both cows showed a decrease in lipin P about the 3rd day of starvation, the decrease becoming more marked in the later stages particularly with the second animal. Similar results have been recorded for the dog [Ling, 1931], pigeon [Müller, 1929], rat [Sure *et al.*, 1933], and contrary results for the rabbit [Iwatsuru, 1925].

There also appears to be some tendency in these two experiments for the sterol to diminish, but the series is not without anomalous values.

With regard to iron, sugar and amino-N, the deviations from the initial values were somewhat larger than the standard deviations of Exp. 1, but no general trend was shown except perhaps in the long starvation, Exp. 4, where the sugar level may have reached a stable value. The variations in Cl content found in Exp. 4 were very small.

Phosphorus distribution. The variations in inorganic and organic acid-soluble P are shown in Tables II and III. From Table II it will be seen that the inorganic P increases regularly during the starvation period, the maximum value being reached 44 hours after realimentation. The changes in organic acid-soluble

P are approximately parallel. Blatherwick [1920] in a single 7-day fasting experiment on a cow observed slight increases in the total and inorganic P contents of the plasma. These he ascribed mainly to the mobilization of mineral reserves to assist in the maintenance of tissue neutrality. No evidence of acidosis was found. Gato [1922] in experiments on fasting rabbits also records changes in blood P. Increases in the inorganic P of the blood during starvation have also been observed by Cavins [1924] and by Wilder [1929] working on rachitic rats.

The organic acid-soluble P increases fairly regularly throughout fasting to a maximum observed about 20 hours after the first post-fasting meal, but the changes are not very great when referred to the standard deviations of Exp. 1. In the present experiments the range found is 3·7–7·5 mg. per 100 ml. blood for inorganic P, 2·2–3·6 for organic acid-soluble P and 6·0–10·8 mg. for total acid-soluble P.

It is of interest to discuss the reason for these increases in inorganic and organic acid-soluble P and also the source of the excess P. During starvation it is well known that extensive tissue breakdown occurs for, in addition to the loss of protein due to the normal wear-and-tear of the organism, energy requirements have to be met, in part at least, from body protein. Oxidative degradation of the phosphoproteins will throw simpler forms of P into the blood-stream and if this is not excreted as rapidly as it is produced, it will be reflected in high levels of the P components of the blood. In addition to the protein of the tissues, the creatinephosphate which is known to be present in muscular tissue is a possible source of organic acid-soluble P.

A second and equally important source of P may be found in the bone phosphates. This is a factor which cannot be ignored when it is remembered that 90 % of the total P of the body is in the skeletal tissue. Utilization of this store has frequently been referred to, particularly in lactating cows. The dephosphorylation of bone would, of course, be accompanied by a mobilization of Ca. Three methods of approaching this problem present themselves. (1) If protein degradation is associated with the rise in blood P, inorganic sulphate in the blood might be expected to show a similar and related increase. Data regarding the inorganic sulphate content of the blood might then be of value. (2) If demineralization of bone occurs during fasting simultaneous increases of blood P and Ca might be observed if no compensatory mechanism is involved. (3) Information about the normal metabolism of the ruminant may be most readily acquired by the examination of the excreta. Extensive catabolism either of bone or of soft tissue ought to be revealed by determination of the output of N, P and Ca.

The second method was followed in experiments subsequent to Exp. 2 and the results are reported in Table III. Concurrently the third method was adopted and the results are reported in Part II.

Ca and P distribution in Exps. 3 and 4. A scrutiny of the results for blood Ca (Table III) shows that there are no consistent changes throughout the starvation period, although the range of variation is rather wide. Thus if demineralization of bone does take place during fasting there must be an efficient mechanism securing the maintenance of the normal blood Ca level.

As before, increases in inorganic P and total acid-soluble P may be observed during fasting, the maximum values being attained on the 6th day in Exp. 3, and on the 5th day in Exp. 4. The increases are not so marked as those of Exp. 2, but evidence that the maximum values are genuine was obtained from the metabolic studies which will be reported later. The smaller increases may be due to the fact that the three animals were in different physiological states. Cow No. 2 was in the 6th month of gestation whereas the others were non-pregnant. The pregnant fasting animal has not only to obtain energy for its own maintenance but has also to provide energy and nutriment for the growth of the foetus. The foetus will require not only fat, protein and carbohydrate for its growth, but also minerals, and the requirements of the latter (especially of Ca and P) will be very high from the 5th month onwards [Turner, 1931]. From this point of view, and in the absence of new sources of P, a tendency for the blood P of the maternal circulation to decrease rather than increase might be expected. Some other factor such as extensive catabolism of P-containing material would therefore seem to be involved.

II. CALCIUM AND PHOSPHORUS METABOLISM DURING FASTING, AND DURING REALIMENTATION FOLLOWED BY FASTING.

The object of this part of the work was to study the Ca and P excretions during fasting in relation to the blood P in the hope that such an investigation might reveal the cause of the increased mineral metabolism during starvation. The urinary analysis of Benedict's fasting experiment [Benedict & Ritzmann, 1927; Carpenter, 1927], although fairly complete with respect to N and S, contained only a few P analyses. Determinations were made of fixed bases but not of Ca, and the analyses of faeces during fasting were confined to dry matter and to total N. In the present paper the rebuilding of both the protein and the skeletal tissue has been followed, while the relationship between blood inorganic P levels and urinary P excretion has also been investigated. The work is divided into three sections.

SECTION A.

During Exps. 3 and 4 already described, urinary and faecal P and faecal Ca were estimated. The results are set out in Table IV.

Table IV. *Ca and P excretion (g./day).*

		Exp. III				Exp. IV			
		Urine		Faeces		Urine		Faeces	
Period	Days of fast	P	N/P	Ca	P	P	N/P	Ca	P
Prefasting	—	—	—	—	—	0·07	313	14·69	4·53
	—	—	—	—	—	0·07	265	13·99	5·71
	—	—	—	—	—	0·06	248	14·08	5·72
	—	0·04	359	9·72	5·45	0·09	233	13·44	6·72
	—	0·10	108	12·82	4·53	0·08	244	7·37	6·43
	—	0·10	155	13·08	4·81	0·12	222	12·13	7·22
	—	0·07	185	11·17	5·13	0·13	200	11·70	5·85
	—	0·09	133	19·46	7·34	0·06	233	9·83	6·00
	—	0·09	103	11·75	4·34	0·09	286	8·01	5·50
Early fasting	1½	0·14	106	13·16	5·88	0·11	179	14·82	6·84
	2½	0·18	110	4·39	2·25	0·09	378	7·85	3·59
	3½	0·14	142	5·04	1·98	0·07	609	5·35	4·17
	4½	0·13	220	3·08	1·10	0·13	322	5·62	2·80
Effective fasting	5½	0·48	57·4	2·68	0·88	0·23	152	2·65	2·76
	6½	2·81	17·6	2·66	0·85	0·48	67·1	1·29	1·09
	7½	4·46	12·1	3·61	0·91	2·46	16·3	3·44	4·00
	8½	—	—	—	—	4·27	10·9	13·48	4·60
	9½	—	—	—	—	3·99	11·0	4·64	3·71
	10½	—	—	—	—	3·72	11·6	3·12	4·01

The Ca and P intakes in the prefasting period were 14·0 and 6·90 g./day respectively in both experiments.

It is of interest to observe that the urines of the cows in the four fasts remained alkaline. This was contrary to the results of Carpenter [1927] where the urine of steers though initially alkaline became acid after a few days.

Urinary P. The P percentage and total daily excretion of P in the urine are remarkably low in the preliminary period (low protein diet) and during the first 4 days of starvation. The P concentration in the urine never exceeds 5 mg./100 ml. and the total daily output is always less than 200 mg. Thus from the point of view of P excretion fasting is commenced from a definite equilibrium with a definite basal urinary output. These low figures are similar to those obtained by Meigs and his co-workers [1919; 1926], and much lower than those found for the human subject. The urinary P output of man, although largely dependent on the diet, is usually about·2 g. per day. In the calf before rumination begins, the output of urinary P is higher than that of cows. This difference may possibly be associated with the type of digestion and absorption characteristic of ruminants and in this connection it may be mentioned that ruminants also differ markedly from non-ruminants in the distribution of the P compounds in the blood, the organic acid-soluble P fraction of the former being particularly low [Blackwood, 1934]. The low level of urinary P excretion is maintained for the first 4 days of fasting. This is in accord with the general conceptions of ruminant digestion. On the 5th and subsequent days, however, a marked change occurs, characterized by progressive increases in percentage and total output of P. The levels attained are similar to those obtained in carnivorous animals including man where fasting is accompanied by a continuous excretion of P. Benedict [1907; 1915] finds that the P output continues in man even throughout long fasts and at the 31st day in one experiment 1·3 g. P_2O_5 were excreted. Occasionally also, a rise in urinary P was observed during the first few days of fasting. Similar results are recorded by Cathcart [1907]. The source of this continued excretion of P during fasting, whether from bone or muscle tissue, can only be determined by correlation with the N and Ca outputs. The time of the rise in urinary P excretion is coincident with the maximum value of inorganic and total acid-soluble P in the blood. This suggests a close relation between the two phenomena. A proportionality between plasma phosphate and urinary phosphate excretion has been postulated by Addis *et al.* [1925] under certain restricted conditions. The blood P therefore appears to increase to a limiting value, after which increased urinary excretion commences.

Correlation of N and P outputs in urine.[1] If muscle protein alone is the source of the urinary P then one would expect the ratio of urinary N to P found, after the exogenous output of these elements has ceased, to approximate to that of muscle itself, namely 17·4 [Lusk, 1931]. The ratio found in the experiments is given in Table IV. The original value of the ratio is seen to be very high and these high values are maintained during the first 4 days of starvation. An extraordinarily rapid fall then takes place. In Exp. 3, owing to the shortness of effective fasting, no equilibrium is attained. The ratio, however, on the last day of the fast is 12·1. In Exp. 4, equilibrium is established at a value approximating to 11·2 from the 8th day.

It is clear then that catabolism of some P-containing material in addition to protein has taken place. Similar low ratios have been found by Cathcart [1907] and other authors and have been generally ascribed to demineralization of bone. A further explanation might rest in the fact that the urine may not be the only excretory channel for P during starvation. Before discussing this question, however, the figures for faecal Ca and P must be considered. It may be noted here that no urinary Ca determinations are recorded since previous experience and subsequent experiment show that such excretion is normally negligible and shows no rise during starvation.

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[1] The N metabolism of these fasts has been included in a paper by Hutchinson & Morris [1936] from which we quote the figures for urinary N.

Faecal P and Ca. During the preliminary period of Exps. 3 and 4 the daily faecal excretion of P represents a fairly uniform basal level, while the percentage composition of the dry faeces shows remarkably small variations. In Exp. 3 this percentage composition (*ca.* 200 mg.) is maintained throughout the entire fasting period. The daily output, however, decreases rapidly to an equilibrium level of about 0·9 g. In Exp. 4 the percentage composition of the dry faeces rises rapidly during starvation. In the early days of starvation a fall in faecal output of dry matter is accompanied by a decrease in the faecal P output. Thereafter, however, the P output rises sharply and almost reaches the prefasting level. The salient feature of each experiment is, then, the continued faecal excretion of P during starvation. In the later stages of fasting this P can only arise from endogenous sources.

The Ca percentage in the dry faeces in each experiment shows no marked difference between the prefasting and starvation periods. The total output, however, although falling rapidly from the prefasting level never becomes insignificant. Thus in the last 3 days of Exp. 3, 8·95 g. of Ca are eliminated and in the last 6 days of Exp. 4, 28·62 g. This, too, can only be of endogenous origin.

This continued excretion of Ca and P in starvation faeces has also been observed by Wellmann [1908] in rabbits.

The main source of the endogenous Ca excretion must be the bone. Such demineralization must result in a simultaneous liberation of P so that part, at least, of the P excreted either in the urine or faeces must arise from this source. The presence of P derived from bone in the urine, in addition to that from protein, is an adequate explanation of the low N/P values observed in the urine.

It has been accepted for some time that Ca can be eliminated from the blood-stream by re-absorption in the lower intestine. Excretion of Ca by the kidney can only take place in the form of soluble salts [Holt *et al.*, 1925]. This excretion will be governed partly by the solubility products of the salts themselves and partly by the reaction of the urine. The urine of the cow is normally highly alkaline and this fact explains why simultaneous large excretions of Ca and P do not take place via the kidney. Such simultaneous eliminations are necessary during fasting, and in view of the maintenance of the alkalinity of the urine in these experiments, one can only assume that re-excretion of both Ca and P into the bowel is possible. This is in agreement with the views of Shelling [1932].

Phosphorus balance sheet. (*a*) *Protein.* Assuming that the urinary N in the later stages of the fast is entirely endogenous and derived from body protein, the P from the same source may be calculated. P derived from protein $= \frac{\text{urinary N}}{17\cdot4}$ where $17\cdot4 = \frac{\text{N}}{\text{P}}$ in muscle. (*b*) *Bone.* It is generally accepted that 85 % of the Ca of bone is present as calcium phosphate ($Ca_3(PO_4)_2$), the remainder being mainly carbonate [Shear & Kramer, 1928]. The amount of Ca found in the soft tissues of the body is usually very small. Assuming as before that the Ca in the later stages of the fast is derived from bone, then Ca derived from bone phosphate = total Ca excreted × 0·85, and so P derived from bone $= \frac{\text{total Ca} \times 0\cdot85}{1\cdot93}$ where $1\cdot93 = \frac{\text{Ca}}{\text{P}}$ as in $Ca_3(PO_4)_2$. Making these calculations on the data of the fasts from the fourth day onwards, part of Table V was drawn up.

Table V. *P balance sheets for effective fasting period* (*g./day*).

Exp.	Days of fast	Urinary N	P from protein	P from bone	Total P excretion by calculation	Total P excretion actually found
III	5½	27·32	1·86	1·18	3·04	1·36
	6½	49·45	3·36	1·17	4·53	3·66
	7½	54·12	3·68	1·59	5·27	5·37
				Total	12·84	10·39
IV	5½	34·98	2·38	1·17	3·55	2·99
	6½	32·21	2·19	0·57	2·76	1·57
	7½	40·76	2·77	1·51	4·28	6·46
	8½	46·71	3·18	5·94	9·12	8·87
	9½	43·96	2·99	2·04	5·03	7·72
	10½	42·76	2·91	1·37	4·28	7·73
				Total	29·02	35·34
V	5	35·28	2·03	0·76	2·79	1·54
	6	67·94	3·90	0·52	4·42	3·96
	7	23·59	1·36	0·16	1·52	0·99
	8	32·34	1·86	0·47	2·33	1·65
	9	25·58	1·47	0·55	2·02	3·06
				Total	13·08	11·20
VI	5	52·02	2·99	2·55	5·54	2·97
	6	48·18	2·77	2·07	4·84	2·27
	7	49·49	2·84	1·31	4·15	2·58
	8	None	None	2·07	2·07	0·45
	9	65·50	3·76	1·11	4·87	4·97
	10	100·30	5·76	1·68	7·44	7·51
	11	None	None	5·11	5·11	0·33
	12	117·40	6·75	2·69	9·44	9·66
				Total	43·46	30·74

The total P excretion calculated is in fairly close agreement with that found and this is a test of the validity of the factors used and of the assumptions made. We regard this therefore as strong evidence that during prolonged fasting continuous mobilization of bone and protein P takes place.

SECTION B.

After the completion of the fasts in Exps. 3 and 4, the cows were placed on a high protein diet until N equilibrium was established. A low protein ration was then given followed by the original high protein diet, the change over being again determined by the attainment of N equilibrium values. The P intakes on the high and low protein diets were 15·6 and 8·9 g./day respectively. Blood samples were taken from alternate mammary veins at intervals and the P distribution determined. The results are given in Tables VI and VII.

Blood levels. Each cow finished the fasts of Exps. 3 and 4 with comparatively low blood levels of inorganic P, namely 3·82 mg. and 3·74 mg. The immediate response to a high protein diet was a large increase in this level, the mean values during this period being 5·23 and 5·35 mg. The organic acid-soluble P showed no significant change. In the low protein period levels prevail with means of 4·53 mg. and 4·35 mg. In the subsequent high protein period little change in these values is observed. The experiments of Robinson & Huffmann [1926] and Huffmann *et al.* [1933] have shown that the amount of P in the diet has a marked influence on the P content of the blood. Two possibilities present themselves. (1) The differences in the daily P intakes 15·64, 8·90 and 15·64 g. may not be sufficiently great to bring about the changes noted by these authors. Our figures were, of

Table VI, Exp. III. *P excretion, and also P distribution in the blood, on high and low protein diets.*

Diet and P intake	Days	P excretion (g./day) Faeces	Urine	Retention	P in the blood (mg./100 ml.) Inorganic	Organic acid-soluble	Lipin
High protein 15·64 g. P per day	1	26·49	2·33	-13·18	—	—	—
	2	16·89	2·05	-3·30	—	—	—
	3	13·86	1·93	-0·15	—	—	—
	4	8·02	1·13	6·49	6·58	1·76	9·36
	6	11·19	1·32	3·13	5·48	2·46	9·60
	8	12·38	0·61	2·65	4·60	2·61	9·28
	9	11·24	0·39	4·01	—	—	—
Low protein 8·90 g. P per day	10	9·41	0·34	-0·85	4·28	1·81	9·76
	12	14·42	0·17	-5·69	4·33	2·31	9·76
	14	6·30	0·28	2·32	4·85	2·42	9·92
	16	4·73	0·21	3·96	4·35	2·59	10·16
	18	7·86	0·17	0·87	4·49	3·12	10·00
	20	5·07	0·19	3·64	4·93	2·75	10·01
	22	8·61	0·11	0·18	4·45	2·78	10·00
	24	7·24	0·14	1·52	4·42	3·28	9·74
	26	9·44	0·10	-0·64	4·52	2·43	9·77
	28	5·18	—	3·72	4·46	2·23	9·93
High protein 15·64 g. P per day	29	5·47	0·18	9·99	4·55	3·00	10·39
	31	8·21	0·19	7·24	4·85	1·56	9·00
	33	7·22	0·12	8·30	—	—	—
	35	8·60	0·07	6·97	4·28	2·58	—
	36	9·68	0·08	5·88	4·45	—	8·83
		Mean values:	High protein		5·56	2·28	9·41
			Low protein		4·51	2·57	9·91
			High protein		4·53	2·38	9·41

Table VII, Exp. IV. *P excretion, and also P distribution in the blood, on high and low protein diets.*

Diet and P intake	Days	P excretion (g./day) Faeces	Urine	Retention	P in the blood (mg./100 ml.) Inorganic	Organic acid-soluble	Lipin
High protein 15·64 g. P per day	1	0·60	0·12	14·92	5·85	2·06	9·69
	3	8·66	0·12	6·86	5·21	1·80	9·60
	5	4·43	0·11	11·10	5·30	1·92	9·54
	7	7·06	6·23*	2·35	5·90	2·09	9·65
	9	8·69	0·11	6·84	5·00	2·39	9·71
	11	11·46	0·10	4·08	5·80	2·94	9·47
	13	5·61	0·13	9·90	5·26	3·00	9·64
	15	8·46	0·09	7·09	5·25	3·13	9·51
	17	11·64	0·08	3·92	5·32	3·07	10·11
	19	8·65	0·09	6·90	4·62	3·27	9·72
	20	7·92	0·07	7·65	—	—	—
Low protein 8·90 g. P per day	21	7·39	0·08	1·43	4·80	3·08	10·05
	23	10·13	0·14	-1·37	4·74	2·85	9·82
	25	7·90	0·06	0·37	4·50	2·51	10·48
	27	11·07	0·11	-2·18	4·34	2·66	9·53
	29	9·23	0·10	-0·43	3·90	2·74	—
	31	6·41	0·10	2·39	3·90	2·96	9·36
	33	5·89	0·07	2·94	4·29	2·55	9·72
High protein 15·64 g. P per day	34	8·00	0·13	7·51	4·28	2·20	9·80
	36	8·21	0·17	7·26	3·82	2·96	10·88
	37+38	14·15	0·09	1·40	5·15	2·62	9·16
	40	8·48	0·11	7·05	4·45	2·56	10·84
	42+43	20·96	0·14	5·09	4·43	3·17	11·60
	44+45	24·06	0·18	3·52	—	—	—
		Mean values:	High protein		5·35	2·56	
			Low protein		4·35	2·77	9·83
			High protein		4·41	2·70	10·46

course, regulated by the protein level desired. (2) The previous nutritional state of the animals was such that the claims of replacement of body tissue would be paramount.

Urine. During the high protein period of Exp. 3 urinary P is excreted at a high level. Progressive decreases in percentage composition and total output are noted and at the end of the period a normal excretion level is attained. This may represent a descent from the high levels observed during fasting. A remarkable constancy in these levels persists throughout the subsequent periods. In Exp. 4 a similar low level prevails even throughout the initial high protein period. The value marked with an asterisk in Table VII is inexplicable.

Faeces. The total P output in the faeces is somewhat irregular but usually no very great variations occur. The values in the high protein period of Exp. 3 are above those observed in the subsequent high protein period.

Retention. In Exp. 3 satisfactory retention of P was not established until the second period of high protein diet. In Exp. 4 the first high protein level was sufficient to establish a positive P balance. This, however, could not be maintained on the low protein diet but was re-established on changing to the high protein level.

Probably a number of factors are involved in the restoration of bone tissue, including the amounts of Ca and P absorbed, the ratio of assimilated Ca and P, the excretion of these elements and the physico-chemical conditions prevailing at the site of deposition of bone substance. The delayed or unsatisfactory realimentation observed may be due to failure to satisfy the requirements of all of these factors. In addition, phosphoprotein must also be restored.

SECTION C.

An opportunity was afforded by the continuation of the fasting experiments already described to make a further study of the P metabolism. The two cows were put out to grass following realimentation. They were then fasted for further periods of 10 and 12 days respectively. The results are reported in Table VIII (Exps. 5 and 6).

Blood and urinary P. It is at once apparent that neither cow started the fast with a normal level of blood inorganic P, the initial values being 7·48 mg./100 ml. and 6·50 mg./100 ml. These extremely high values may be associated with the prefasting diet of fresh young grass, which, being rich in minerals, would secure effective realimentation, and it was not to be anticipated that fasting could result in the production of higher levels. It was therefore thought of interest to continue the study of blood and urinary P in these somewhat unusual cases with the object of testing the validity of the views already expressed on the relation of these two P levels.

The high blood P level is maintained for the first 4 days of fasting and is accompanied by a fairly high level of urinary P. This is especially noticeable in the percentage composition of the urine.

Thereafter with Exp. 6 somewhat lower and normal levels of blood P prevail, variations about the mean value of 4·99 being small. The urinary excretion of P, however, rises in precisely the same fashion as in the earlier fasting experiments. On the final day of the fast the P excretion reached the abnormally high value of 9·6 g. while the percentage composition was 141 mg./100 ml. This rise in urinary P again illustrates the degradation of body P. The efficiency of the renal excretion throughout the entire fasting period has thus prevented any temporary accumulation of inorganic P in the blood, so that no rise in level comparable with

Table VIII. *Blood P and excretion data during 10½-day and 12-day fasts.*

		Blood P (mg./100 ml.)				Ca and P excretion (g./day)			
						Urine		Faeces	
Exp.	Days of fast	In-organic	Organic, acid-soluble	Lipin	Day of fast	P	Ca	P	Ca
V	1½	7·48	4·50	9·38	1	0·33	0·14	4·73	21·98
					2	1·89	0·25	5·18	11·00
	3½	7·65	4·04	9·22	3	0·78	0·10	1·58	2·72
					4	1·04	0·07	1·46	1·82
	5½	4·56	3·69	10·46	5	0·22	0·04	1·32	1·69
	6½	3·79	4·87	9·46	6	2·90	0·09	1·07	1·10
	7½	3·77	4·47	9·82	7	0·44	0·18	0·56	0·19
	8½	3·26	5·20	9·97	8	0·32	0·14	1·33	0·92
	9½	2·62	4·53	10·43	9	0·14	0·08	2·92	1·16
	10½*	4·43	4·35	12·60	—	—	—	—	—
VI	1½	6·50	4·09	—	1	2·95	0·31	1·34	18·20
					2	1·26	0·07	0·69	10·50
	3½	7·55	4·25	—	3	1·17	0·10	1·42	15·08
					4	1·68	0·07	0·37	4·44
	5½	5·34	3·86	—	5	2·38	0·25	0·60	5·54
	6½	4·86	3·67	—	6	1·81	0·19	0·46	4·51
	7½	4·70	4·06	—	7	2·24	0·03	0·34	2·95
	8½	5·17	4·33	—	8	None	None	0·45	4·70
	9½	4·12	3·54	—	9	4·58	0·11	0·39	2·41
	10½	5·08	3·88	—	10	7·04	0·13	0·47	3·68
	11½	4·92	3·62	—	11	None	None	0·33	1·16
	12½	5·10	3·97	—	12	9·59	0·16	0·07	0·45

* Jugular post mortem sample.

those of the earlier fasts occurs. It is also noticeable that the fall in blood P level is coincident with the marked rise in the urinary P percentage and this points to the establishment of an equilibrium at the renal tubules. Further consideration of the urinary P of Exp. 5 will be given later.

Ca excretion. It has already been noted that urinary Ca is invariably low. This has been determined throughout the two fasts recorded and shows no significant change. The faecal Ca output declines from its initial high values, a result which may be due to grass feeding, but the output is maintained at a steady level, indicating again a continuous mobilization of bone tissue.

Faecal P. Faecal P output is also maintained at an appreciable level even in the later fasting period, although decreasing from its original figure. If a balance sheet is struck of the mobilization of P reserves in the body on the lines already indicated the part played by protein and bone respectively in the maintenance of bodily activity may be calculated. The data of Table V pertaining to the present experiments have been drawn up on these lines. The agreement between the calculated and observed values of P excretion is good in Exp. 5 but in Exp. 6 a low urinary output, suggesting a suppression of urine and a formation of urinary calculi in the later days of the fast, leaves a fairly large proportion of the expected P excretion unaccounted for. Whilst protein is seen to contribute the larger part of the P excreted, the utilization of the skeletal tissue P is remarkably rapid in onset and its magnitude is not inconsiderable. One interesting feature of these

Exp. no.	P derived from protein g./day (mean)	P derived from bone g./day (mean)
3	2·97	1·31
4	2·85	2·10
5	2·12	0·49
6	3·11	2·32

balance sheets is the agreement in three of the fasts between the relative utilization of protein and bone P and the dissimilarity of the fast of Exp. 5.

Exps. 3 and 5 were carried out on one animal and Exps. 4 and 6 on another. In Exp. 3 there is a suggestion that the mineral reserve was not capable of mobilization to the extent seen in Exps. 4 and 6 and in the second fast with this animal the failure of bone mobilization is most evident. This failure was characterized by marked symptoms of tetany, necessitating the destruction of the animal, and if reference is made to Table VIII the clinical picture is one of very low blood inorganic P accompanied by a diminished urinary P excretion. Presumably investigation would also have shown a low blood Ca.

It appears then that during fasting effective and continuous mobilization of Ca and P from skeletal tissue is necessary, not only to maintain the level of these constituents in the blood but also to aid in the maintenance of tissue neutrality, to preserve a renal equilibrium and possibly to facilitate the conversion and transport of body reserves to meet energy requirements.

GENERAL SUMMARY.

A study of various constituents of blood during fasting and after meals of varying fat content is reported. With the exception of lipin and of acid-soluble P, no constituent showed any consistent change in level. Lipin P was decreased by effective periods of fasting, whilst inorganic and organic acid-soluble P were markedly increased, indicating extensive catabolism of body P. The source of this P is discussed. It is concluded that, apart from the two types of P, the level of the blood constituents investigated is not affected in the ruminant by the processes of digestion and absorption.

Continuous excretion of Ca and P was found during effective fasting of ruminants. The blood P level is correlated with the urinary P output. The source of the continuous excretion of P is endogenous and is shown to be derived from bone and phosphoprotein material. The necessity for this dual mobilization is discussed and an interesting case of failure to maintain effective mobilization is reported.

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CCLVI. THE MICRO-DETERMINATION OF GLYCINE IN PROTEIN HYDROLYSATES.

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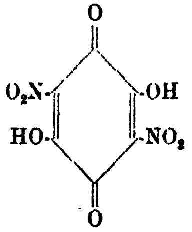
The separation of the amino-acids obtained on protein hydrolysis into three fractions by means of the differing solubilities of their copper salts as indicated in previous publications [Town, 1928; 1936] is generally satisfactory in that the amino-acids are largely found in those fractions to be expected from the known properties of their copper salts. Overlapping due to the formation of mixed copper salts and to changes in the solubility of the copper salts due to the presence of other soluble salts occurs only to a very small extent.

Although however this separation into large fractions is workable, separation of the individual amino-acids by fractional crystallization is very difficult. The author has therefore been carrying out concurrently a search for specific precipitants for some of the amino-acids. In particular, substances analogous to flavianic acid have been studied, all being aromatic compounds containing the *o*-nitrophenolic grouping. The only success was obtained in the case of nitranilic acid which is 2:5-dihydroxy-3:6-dinitro-*p*-benzoquinone of the formula:



Nitranilic acid acts as a strong dibasic acid and forms a salt with glycine of the formula $(C_2H_5O_2N)_2.C_6(OH)_2(NO_2)_2O_2$. This salt is soluble in water to the extent of 0·8 %, but is practically insoluble in 80–100 % alcohol. The acid gives no precipitate with alanine, valine, leucine, phenylalanine, glutamic acid or tryptophan, although with the latter the solution becomes very dark. Glycine nitranilate crystallizes in large yellow octahedral crystals which darken rapidly on heating to 120°; it is therefore dried *in vacuo* over H_2SO_4. Nitranilic acid solutions decompose readily on standing, so that in forming the derivative the requisite amount of acid is weighed out, dissolved in a few ml. of alcohol and added to the solution to be precipitated.

As indicated below the precipitant has been used to separate an artificial mixture of glycine and alanine, and also to estimate the glycine in gelatin and in caseinogen, these two proteins being chosen as respectively rich and poor in glycine. A solution containing 100 mg. glycine and 162 mg. alanine was separated to the extent of 98 % by treatment with this precipitant. The presence of mineral acids has no harmful effect and the amino-acids are conveniently dissolved in alcohol in the form of their hydrochlorides.

In applying the process to the estimation of glycine in protein hydrolysates, it is important to ensure the absence of inorganic cations, among which Na, K, NH_4, Ca and Ba all form sparingly soluble nitranilates. Once these interfering substances are removed it is possible to estimate the glycine content of a protein using only 200–300 mg. of material.

Determinations carried out on gelatin and caseinogen gave the following results:

	% of glycine in protein	
	As nitranilate	Accepted figures
Gelatin	25·9	25·5
	25·3	—
Caseinogen	3·73	0·45
	3·40	—

In the case of gelatin an attempt was made to isolate glycine from the nitranilate as its 3:5-dinitrobenzoyl derivative according to Saunders [1934] and a crude yield of 82 % was obtained, the yield of pure substance being 27 %. This confirms that it was actually glycine that was precipitated from the gelatin by the nitranilic acid. The figures quoted for gelatin are those of Dakin [1920] and for caseinogen those of Foreman [1919]. In view of the utility of nitranilic acid as a specific precipitant for glycine, the preparation and investigation of substances of analogous constitution is being undertaken.

EXPERIMENTAL.

Preparation of nitranilic acid. 1:4-Diacetoxybenzene (20 g.; by acetylation of quinol) was treated very slowly with fuming HNO_3 (about 10 ml.) at a temperature not exceeding 5° and the resulting solution added very slowly to 70 ml. fuming HNO_3 at 2° with continuous mechanical stirring and maintenance of the temperature below 5°. This process occupied about an hour. Conc. H_2SO_4 (65 ml.) was then added dropwise with stirring, below 5° and during about 3 hours. On continued stirring for a further hour the nitranilic acid crystallized out. The mixture was poured into 10 vol. of ice in a beaker, cooled in ice-salt, and the crystals were collected rapidly on a sintered glass funnel. The crude substance was dried *in vacuo* over H_2SO_4 and KOH and recrystallized from boiling ethyl acetate; yellow prisms; yield 10·6 g.

Separation of a mixture of alanine and glycine. 162·2 mg. alanine and 100·8 mg. gylcine were treated with 1 ml. conc. HCl and 12 ml. water, and the solution was evaporated to 4 ml.; 25 ml. absolute alcohol were added and then 300 mg. nitranilic acid in 5 ml. absolute alcohol. A fine yellow precipitate formed almost immediately, but was kept in the ice-chest for several hours. The precipitate was filtered off, washed with alcohol and dried over H_2SO_4 *in vacuo*; yield 247·8 mg., corresponding to 98 % recovery.

Estimation of glycine in gelatin. 7·079 g. Coignet's gold label gelatin were kept overnight with 100 ml. conc. HCl and then refluxed for 24 hours. The solution was evaporated to dryness on a water-bath to remove excess HCl, the residue dissolved in water and made up to 250 ml. 10 ml. of this solution (equivalent to 283·2 mg. gelatin) were treated with 15 ml. baryta (0·328 *N*) to make it distinctly alkaline to phenolphthalein and air was then drawn through the solution for 1 hour in a water-bath at 70° to remove ammonia. The liquid was just acidified to Congo red by addition of 2 ml. of 4N H_2SO_4, the $BaSO_4$ filtered off and washed and the filtrate and washings evaporated to 4 ml. Absolute alcohol (30 ml.) was now added and a further slight precipitate of $BaSO_4$ filtered off. To the clear alcoholic solution 300 mg. nitranilic acid in 5 ml. of alcohol were added and the whole kept overnight. A well crystallized precipitate formed almost immediately, which after due time was filtered off, washed with absolute alcohol, dried *in vacuo* over H_2SO_4 for 24 hours and weighed. Yield 181·6 mg. A second experiment gave 185·7 mg. These figures correspond to yields of glycine of 25·35 and 25·9 % respectively.

Estimation of nitranilic acid in the glycine nitranilate. 122·4 mg. of the glycine nitranilate obtained above were dissolved in 35 ml. cold water (nitranilic acid decomposes readily in hot solution), and 2 ml. N $BaCl_2$ added: a yellow crystalline precipitate of barium nitranilate immediately separated in shining plates. After some hours the precipitate was filtered off, washed with alcohol, dried at 100° for an hour, cooled and weighed. The weight of barium nitranilate was 116·8 mg. corresponding to a nitranilic acid content of 60·0 % (calc. 60·5 %). 160·1 mg. glycine nitranilate obtained in the second experiment gave 153·2 mg. barium nitranilate corresponding to 60·2 % of nitranilic acid. These figures indicate that the material precipitated by the nitranilic acid from the hydrolysed gelatin is essentially glycine.

Isolation of 3:5-dinitrobenzoylglycine from the glycine nitranilate. The filtrate from the barium nitranilate obtained from the 122·4 mg. glycine nitranilate used above was treated with 0·8 ml. 4N H_2SO_4 to remove excess Ba, and the filtrate from the $BaSO_4$ was evaporated to 5 ml. NaOH was added till the solution was just alkaline to phenolphthalein followed by 1·4 ml. N NaOH and 160 mg. 3:5-dinitrobenzoyl chloride and the whole shaken for 5 min. The mixture was now acidified with dil. HCl and the precipitated 3:5-dinitrobenzoyl compound filtered off. Yield 122 mg., corresponding to a crude yield of 82·3 %. The crude material (M.P. 160°) was boiled once with benzene to extract any free 3:5-dinitrobenzoic acid and then recrystallized from hot water, after which it had M.P. 175°; a second crystallization gave 34·7 mg. pure 3:5-dinitrobenzoylglycine, M.P. and mixed M.P. 180°, corresponding to 25 % of the glycine present and again confirming the identity of the material precipitated by the nitranilic acid.

Estimation of glycine in caseinogen. 0·7855 g. caseinogen was hydrolysed by refluxing with 25 ml. conc. HCl for 24 hours; the solution was evaporated to dryness on a water-bath and the residue dissolved in water, filtered from humin material and made up to 25 ml. 10 ml. of this solution treated exactly as described under the hydrolysis of gelatin, yielded 29·7 mg. glycine nitranilate corresponding to 3·73 % glycine in the caseinogen. A second experiment gave 27·0 mg. nitranilate, equivalent to 3·40 % of glycine. These two precipitates contained 61·0 and 62·5 % of nitranilic acid respectively.

SUMMARY.

A method has been evolved for the micro-determination of glycine in protein hydrolysates. The method has been applied to two representative proteins: one, gelatin, rich in glycine, and the other, caseinogen, poor in this amino-acid. The results in the case of gelatin agree fairly closely with those previously recorded, whilst in the case of caseinogen they are much higher than the accepted values. This may be due to the volatility of glycine ester which caused losses in the isolation by the ester method.

The author is indebted to the late Dr W. H. Hurtley for much encouragement during the course of this work. The Government Grant Committee of the Royal Society is thanked for a grant which defrayed the cost of many of the chemicals used in the course of the work.

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CCLVII. THE SEPARATION OF AMINO-ACIDS BY MEANS OF THEIR COPPER SALTS.

II. AN INVESTIGATION OF THE METHYL ALCOHOL-SOLUBLE COPPER SALT FRACTION, AND THE YIELD OF PROLINE FROM GLIADIN.

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Town [1928] outlined a method for the separation of the amino-acids by means of their Cu salts and for the isolation of proline in a state of purity: in the present paper modifications of the method are described and the yield of proline from gliadin as determined by isolation as the picrate is indicated.

Proline was first isolated by Fischer [1901] from the hydrolysis products of caseinogen by the ester distillation method. Later [1910] he improved the method of isolation, extracting the *dl*-proline with alcohol from the alkaline hydrolysate of gelatin and isolating it as the alcohol-insoluble Cu salt. This was the usual method of estimation of proline until Van Slyke suggested that it could be estimated by a determination of the non-amino-N in the alcohol-soluble portion of the amino-acids. This method gives results considerably higher than those of direct isolation; for example, in the case of gliadin, Osborne & Clapp [1906] obtained by isolation a proline content of 7·06 %, whereas later Osborne & Guest [1911] by Van Slyke's method found 13·2 %.

In the course of the present work six hydrolyses, each of 600 g. of protein, have been carried out. In the early hydrolyses, important findings were: (1) proline is difficult to extract with cold absolute alcohol, indeed the pure substance is only sparingly soluble; (2) proline is partly precipitated by phosphotungstic acid and this reagent therefore is not a specific precipitant for the bases [compare Thimann, 1930]; (3) proline can be effectively separated from other amino-acids by means of the solubility of its Cu salt in cold absolute methyl alcohol; (4) proline can be isolated in a pure state from this Cu salt, after decomposition, as the picrate.

A survey of the available literature indicates that the Cu salts of the amino-acids falls into three characteristic groups:

Group 1. Cu salts soluble in methyl alcohol and also in water: Proline, hydroxyproline, *iso*leucine, valine, the "hydroxyvaline" of Schryver and the peptide of phenylalanine and proline isolated from gliadin by Osborne.

Group 2. Cu salts insoluble in methyl alcohol but soluble in water: Glycine, glutamic acid, serine, alanine, arginine, histidine, lysine and also "hydroxyglutamic acid" and the pyrrolidone compounds.

Group 3. Cu salts insoluble in water and in methyl alcohol: Leucine, phenylalanine, tyrosine, aspartic acid and cystine.

A fourth hydrolysis indicated that 98 % of the total N was accounted for in this gross separation of Cu salts. Only the methyl alcohol-soluble fraction of the latter is considered in this paper. This fraction from the fourth hydrolysis yielded pure proline picrate by the earlier method [Town, 1928].

The methyl alcohol-soluble Cu salt fraction from the fifth hydrolysis (500 g. protein) was decomposed and the proline removed as its picrate (95 g.), the mother liquor being extracted with ether.

The *ether-insoluble fraction*, which still contained picric acid, was freed from the latter and reconverted into Cu salts which were extracted with methyl alcohol; the insoluble portion was rejected. The soluble portion was decomposed and the resulting alcoholic solution treated with alcoholic $CdCl_2$ to precipitate any residual proline. The filtrate, freed from Cd, was saturated with HCl gas and filtered from a small quantity (5 g.) of glutamic acid hydrochloride; removal of HCl left a syrup which contained no proline and from which nothing could be isolated.

The *ether-soluble fraction* was also converted into Cu salts and extracted with dry methyl alcohol. The alcoholic solution of the amino-acids recovered from the soluble Cu salts was precipitated with alcoholic $CdCl_2$ to remove traces of proline. The filtrate, after various unsuccessful attempts to precipitate it with nitro-compounds analogous to picric acid, was dried *in vacuo* and extracted with acetone in a Soxhlet apparatus. The large soluble portion contained diketopiperazines and was left for future examination. The acetone-insoluble residue gave Cu salts consisting of a very soluble minor fraction and a major fraction sparingly soluble in water; from the latter, after decomposition and fractional crystallization of the amino-acids from alcohol, there were obtained 1·43 g. leucine and 3·5 g. *iso*leucine. Confirmation of this isolation will be reported separately [compare Town, 1929].

The total yield of proline obtained in this hydrolysis was 37·7 g.—equivalent to 8·50 % of the dry protein—but it was obtained in three fractions as follows:

	g.
As proline picrate	31·7
As $CdCl_2$ ppt. from ether-insoluble fraction ...	2·7
As $CdCl_2$ ppt. from ether-soluble fraction ...	3·3
	37·7

This yield of proline it is to be noted approximates more closely to that obtained by Osborne & Clapp [1906] by direct isolation than to the figure deduced by Osborne & Guest [1911] from determination of non-amino-N. This hydrolysis however suffered from two important defects. The first was that the separation of glutamic acid as water-soluble Cu salt was incomplete; although only 2·06 % of the glutamic acid came through in the wrong fraction, its presence rendered the isolation of the proline much more difficult. The second defect was that the proline was not completely precipitated as the picrate and also that the picrate has an appreciable solubility in ether; extraction of unprecipitated picrate with ether however removed also much troublesome diketopiperazine material.

The sixth hydrolysis, described in more detail below, was designed to overcome these defects. The dicarboxylates were removed from the H_2SO_4-free hydrolysate as Ba salts insoluble in 75 % alcohol. The filtrate was concentrated at low temperature under reduced pressure to remove ammonia along with the alcohol, freed quantitatively from Ba and then converted into Cu salts. The methyl alcohol-soluble Cu salts were decomposed, the amino-acids taken up in 95 % alcohol and the proline precipitated with alcoholic $CdCl_2$. Only the investigation of the $CdCl_2$ precipitate has been completed and is described here; the filtrate is being examined at present and the results will be communicated later.

The $CdCl_2$ precipitate was decomposed and the aqueous filtrate treated with picric acid, yielding 156 g. proline picrate. The mother-liquor from this yielded ether-insoluble and ether-soluble fractions of which the latter contained, besides picric acid, appreciable quantities of diketopiperazines which are being examined; the ether-insoluble fraction, containing 4·85 g. N, was subjected to Vickery's procedure for the removal of the basic amino-acids which preliminary tests had indicated to be present, after which there was finally obtained a further crop of 7·97 g. pure proline picrate.

The total yield of proline obtained in this hydrolysis is 55·0 g., obtained as follows:

	Wt. of picrate g.	Wt. of proline g.
1st crop	156·0	52·0
Final crops	8·0	2·7
	0·8	0·3
	164·8	55·0

This corresponds to 10·34 % of the dry protein, or expressed as proline N/total N to a yield of 7·32 % of the total N.

It seems important to the author that all results should be expressed in the latter manner, as otherwise unduly favourable figures are obtained for the yields of the protein hydrolysis products. The result obtained above is believed to represent with some accuracy the yield of proline from gliadin. It may be objected that the other fractions of the Cu salts or the $CdCl_2$ filtrate from the methyl alcohol-soluble Cu salt fraction still contain proline. These fractions however have now been examined in sufficient detail to prove the absence of proline; the results of this examination will be reported later. It will be noted that the yield of proline obtained above is higher than that previously obtained by direct isolation, but definitely lower than the figure obtained by merely determining the alcohol-soluble non-amino-N: this is due to the presence of peptides and diketopiperazines some of which are very resistant to hydrolysis. An important feature of this separation is that 94·6 % of the proline was removed in the first crops of picrate; the examination of the residue although occupying several months gave but little to increase the yield: moreover, the picrate obtained was pure and scarcely needed the one recrystallization which it was given.

EXPERIMENTAL.

600 g. gliadin containing 10·22 % moisture, 1·08 % of ether-soluble material and 15·27 % N were boiled under reflux for 36 hours with 30 % H_2SO_4 (510 ml. conc. acid to 2500 ml. water). The total N in the hydrolysed protein was 91·6 g. The hydrolysed solution was diluted with 5100 ml. water and the humin filtered off (8·6 g.; N, 0·3 g.). The filtrate was now treated with hot aqueous baryta until just acid to Congo red, the $BaSO_4$ being filtered and washed twice with boiling water (wt. of $BaSO_4$, 2214 g.; N, 3·02 g.). The filtrate and washings were concentrated to 2870 ml. (N, 88·0 g.) and 786 g. of baryta in hot water added. To the resulting 5 l. of solution, 15 l. 95 % alcohol were added with constant stirring and the bulky precipitate of barium dicarboxylates allowed to settle overnight before filtration. The examination of the dicarboxylates has been completed and will be described later.

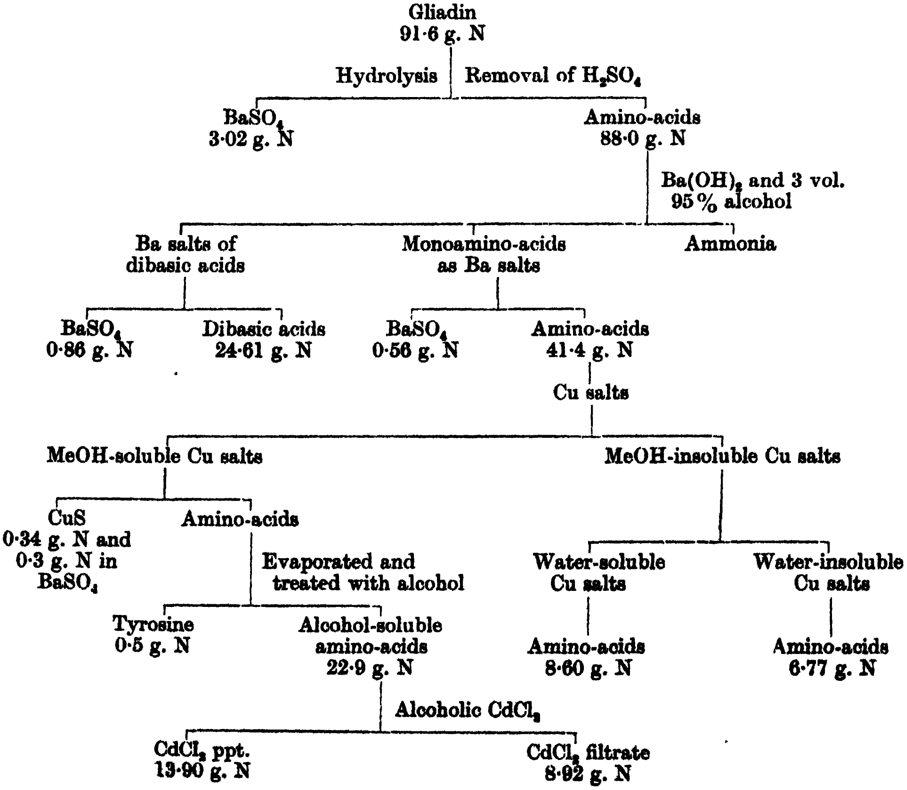
The filtrate and washings from the dicarboxylates were freed from alcohol and ammonia by concentration under reduced pressure (bath at 50°). Removal of ammonia at this stage greatly facilitates the later separation of the Cu salts.

The concentrated solution (4600 ml.) was freed from Ba (wt. of $BaSO_4$, 372 g.; N, 0·56 g.), further concentrated to 4 l. (N, 41·4 g.) and then evaporated on the water-bath with 200 g. of pure $Cu(OH)_2$ until a thick viscous mass was obtained which was granulated with acetone in the manner previously described [Town, 1928]. After complete removal of acetone *in vacuo* over H_2SO_4, the dry salts (565 g.) were extracted six times by shaking with 1·5 l. dry methyl alcohol for 2 hours. The residue was then similarly extracted four times with water. By the above fractionation the amino-acids have been divided up as follows:

	Total N in fraction (after removing metals) g.	% of total N
Dicarboxylates	24·61	26·9
NH_3 distilled from filtrate of dicarboxylates	24·10	26·3
Methyl alcohol-soluble Cu salts	24·82	27·1
Water-soluble Cu salts	8·60	9·4
Water-insoluble Cu salts	6·77	7·4
	88·90	97·1

It will be seen that no serious losses occur in this fractionation, for 97·1 % of the total N has been accounted for, and the remainder is mainly adsorbed on the $BaSO_4$ precipitate.

The methyl alcohol-soluble Cu salts (with which this paper is concerned) were dissolved in water and freed from Cu and the solution of the amino-acids was evaporated to small bulk (N, 24·82 g.). 4 l. 95 % alcohol were now added and 6 g.



of tyrosine which separated were filtered off. The solution was now treated with saturated 95 % alcoholic $CdCl_2$ to complete precipitation. Both precipitate and filtrate were dissolved in water and freed from Cd and Cl. The whole process is summarized diagrammatically above (p. 1840).

Examination of the $CdCl_2$ precipitate.

The aqueous solution (1 l.) of the fraction after removal of the Cd and Cl contained 13·62 g. N of which 26·5 % was amino-N. The solution was treated at boiling point with picric acid (163 g.) equivalent to the non-amino-N present; on cooling the solution set to a paste of crystals of proline picrate; these were collected and recrystallized from hot water. Weight of 1st crop, 138 g.; M.P. 153°. (Picric acid as nitron picrate 66·7 %; theory 66·6 %.)

The filtrate on concentrating to 500 ml. and cooling in the refrigerator yielded a 2nd crop which after recrystallizing amounted to 7·5 g.; M.P. 153°. (Picric acid 66·5 %.) On concentrating the mother-liquor to 200 ml. a 3rd crop was obtained; wt. after washing with ether and recrystallizing 9·0 g.; M.P. 153°. (Picric acid 66·7 %.) Further concentration to 100 ml. gave another 1·5 g. proline picrate the total weight so far obtained thus being 156 g., corresponding to 52·2 g. proline or 6·34 g. N. This is a yield of 9·8 % of the protein or 6·92 % of the total N. The solubility of proline picrate was determined at this stage; 50 ml. of a saturated solution at 26° on evaporation to dryness gave 1·096 g. proline picrate, corresponding to a solubility of 2·2 % at 26°.

The mother-liquor from the picrate was diluted with an equal volume of water and extracted with ether: the aqueous solution was then strongly acidified with H_2SO_4, the precipitated picric acid filtered off (20 g.), and residual picric acid removed by extraction with ether. The final aqueous solution contained 4·85 g. N.

All the ether extracts were mixed, freed from ether, decomposed with an excess of H_2SO_4, picric acid (22 g.) filtered off and the N determined; found 1·67 g. The N of the $CdCl_2$ precipitation thus accounted for is:

	g. N		g.
As proline	6·34	Original N content	13·62
In mother-liquor from proline	4·85		
In ether extracts	1·67		
Loss	0·76		
	13·62		

The ether-extracted material on freeing from H_2SO_4 and evaporation *in vacuo* solidified; it was very soluble in water and gave a slight flocculent precipitate with phosphotungstic acid. Amino-N was 43·6 % of total N and the fraction contained appreciable quantities of diketopiperazines. It has not yet been systematically examined.

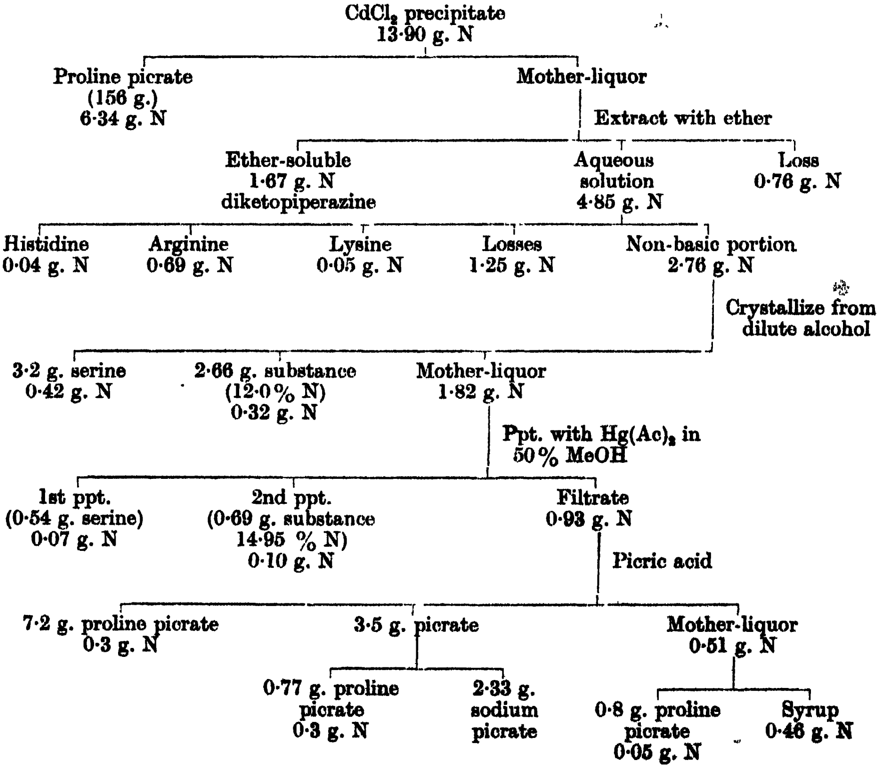
Examination of mother-liquor from proline picrate. This fraction (4·85 g. N) was evaporated to a syrup which was insoluble in acetone and alcohol but soluble in 60 % alcohol. Amino-N was 55 % of total N and was not increased on prolonged hydrolysis with conc. HCl, thus indicating the absence of peptides. The fraction gives no precipitate with alcoholic $CdCl_2$, a small precipitate with mercuric acetate in 50 % alcohol and a heavy precipitate with phosphotungstic acid. It contains no ammonia or tyrosine and gives an amorphous Cu salt soluble in methyl alcohol. When treated according to Vickery & Leavenworth [1928] it yielded 0·68 g. histidine diflavianate, 6·0 g. arginine flavianate equivalent to 2·14 g. arginine and 0·71 g. lysine picrate. The recovery of these bases was accompanied by appreciable losses of N, the nature of which could not be determined.

The residue not precipitated by phosphotungstic acid in the above separation, after freeing from precipitants, contained 2·76 g. N of which 61 % was amino-N. On evaporation it yielded a syrup insoluble in alcohol but soluble in 60 % alcohol; no crystalline picrate could be obtained from it. The solution in 60 % alcohol on standing in the ice-chest deposited 3·20 g. of well crystallized material containing 13·23 % N and identified as serine.

The filtrate from this on evaporating at room temperature deposited 2·66 g. of a material crystallizing in rosettes of needles containing 12·0 % N; M.P. 237°. This material is not valine, because although apparently homogeneous it yields no phenyl*iso*cyanate or 3:5-dinitrobenzoyl derivative. It is being further investigated.

After these substances had separated there was left a solution containing 1·82 g. N of which 50 % was amino-N. After preliminary experiments it was treated with 15 % mercuric acetate in 50 % methyl alcohol; the precipitate after decomposition gave a solution (0·19 g. N) from which after evaporation and treatment with alcohol there was obtained 0·54 g. more of serine.

The filtrate from this precipitation on diluting with twice its volume of 95 % alcohol deposited a further copious precipitate of mercury salt which was collected and decomposed. The solution (0·63 g. N; 68 % amino-N) was evaporated to a syrup from which, by long keeping with methyl alcohol, 0·69 g. of substance crystallizing in tetrahedra was obtained (N, 14·95 %; α-naphthyl*iso*cyanate M.P. 184°). An accident caused the loss of the rest of this fraction.



The residue, not precipitated by mercuric acetate, after freeing from reagent contained 0·92 g. N (36 % amino-N) but gave only a syrup on evaporation. Picric acid equivalent to the non-amino-N was added and the mixture was left in the ice-chest; 7·2 g. proline picrate were obtained having M.P. 149°, raised to 153° after one recrystallization (picric acid 66·5 %). The solution on repeated concentration gave further crops which were fractionally crystallized yielding 0·77 g. proline picrate and 2·33 g. sodium picrate which probably originated from the baryta used in the precipitation of the dicarboxylates.

The mother-liquor from these picrates, freed from picric acid, still contained 0·51 g. N. It was precipitated with methyl alcoholic mercuric acetate and from the non-precipitated portion 0·8 g. proline picrate, M.P. 149°, was isolated. No other identifiable component could be obtained. The results of the above series of operations are indicated in the above scheme (p. 1842).

DISCUSSION.

A balance sheet of the distribution of the N in the $CdCl_2$ precipitate of the methyl alcohol-soluble Cu salts appended below indicates clearly the substances which have been isolated as well as the losses of N which have been incurred.

N distribution in the $CdCl_2$ precipitate.

		N (g.)	Original N content
1st crops of proline picrate	6·34 g.		13·62 g.
Final crops of proline picrate	0·36 g.	6·70	
Diketopiperazine material		1·67	
Histidine		0·04	
Arginine		0·69	
Lysine		0·05	
Losses in pptn. of bases		1·25	
Serine		0·50	
Substance with 12·0 % N		0·32	
Substance with 14·95 % N		0·10	
Loss due to accident		0·50	
In final syrup		0·44	
		12·26	
Unaccounted for; i.e. lost on ppt. etc.		1·36	
		13·62	

Although the losses are high, it must be remembered that this examination was carried out under no standard plan, the method of attack having to be determined at each stage; it has been of an exploratory nature (the results have not been corrected for the material used in the N determinations). Also the examination of the proline fraction is well known to be difficult. The investigation shows that 49·2 % of the N of the fraction is proline-N.

According to the literature the methyl alcohol-soluble Cu salts should be those of proline, hydroxyproline, *iso*leucine, valine and the peptide phenylalanylproline. Of these *iso*leucine and valine are not precipitated by alcoholic $CdCl_2$ and should be in the $CdCl_2$ filtrate. The investigation of this fraction has proceeded far enough to say that these amino-acids are definitely present and the details will be reported later.

The amino-acids serine, arginine, histidine and lysine have come through from the water-soluble Cu salt fraction to the extent of 13·7, 2·0, 8·0 and 29·2 % respectively on the basis of the figures of Osborne *et al.* [1915] for the percentages of these amino-acids present in gliadin: thus the leakages into the wrong fractions are quite small, being serious only in the cases of arginine and serine. These amino-acids also are not the main hydrolytic products of the protein. The investigation of the water-soluble and the water-insoluble fractions has proceeded far enough to indicate that they contain substantially the amino-acids to be anticipated. It therefore appears that the separation of amino-acids by means of their Cu salts may be of some permanent value and perhaps contribute to the elucidation of that fraction of the total N of proteins which is as yet unaccounted for, and which unfortunately is still a considerable proportion of the whole.

SUMMARY.

An investigation is being carried out to determine whether the differences in the solubilities of the Cu salts of amino-acids can be applied to effect their separation.

In the gross separation of the Cu salts into three fractions 97 % of the N can be accounted for.

The examination of the $CdCl_2$ precipitate obtained from the methyl alcohol-soluble Cu salts is described in detail.

The yield of proline from gliadin has been determined by direct isolation as the picrate, this amino-acid being the chief component of the $CdCl_2$ precipitate.

The yield obtained of 10·34% is believed to represent with some accuracy the proline content of gliadin. The figure is considerably higher than that previously obtained by isolation, but much lower than values obtained by determination of the non-amino-N. This is due to the presence of diketopiperazines, which have been isolated but not yet identified.

Other amino-acids, notably arginine and serine, which should belong to the water-soluble Cu salt fraction, find their way into the methyl alcohol-soluble Cu salt fraction to a small extent.

The examination of the $CdCl_2$ filtrate from the methyl alcohol-soluble Cu salt fraction is being continued and will be reported later.

A large part of this work was carried out during the tenure of an Alexander Brown Coxe Research Fellowship at Yale University, Connecticut, U.S.A. The author is indebted to the late Prof. L. B. Mendel and to Dr H. B. Vickery for much helpful advice and encouragement while the work was being carried out in America, and also to the late Dr W. H. Hurtley during the continuation of the work at St Bartholomew's Hospital Medical College. The author also desires to thank the Government Grant Committee of the Royal Society for a grant which defrayed many of the incidental expenses.

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CCLVIII. MICRO-POTENTIOMETRIC TITRATIONS OF NORMAL HORSE SERUM GLOBULINS.

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(*Received 11 July 1936.*)

The measurement of titration curves affords a method of investigating protein solutions which is simple to manipulate and in principle easy to interpret; the interpretation in detail in terms of the dissociation constants of individual groups has not yet been carried out with success, although a semi-quantitative explanation has been given in some cases; it appears likely (for reasons to be published elsewhere) that a detailed interpretation is a matter of great complexity.

For the present purpose interpretation was not the first object; it was required rather to be able by this means to compare the behaviour of different globulins. Identity or otherwise of the titration curves would be a strong argument for the identity or difference of two specimens of protein. It was intended to use this as a companion method to that of spectrographic analysis described by Holiday [1936].

The first object was to establish the titration curves of normal horse pseudoglobulin and euglobulin. As well as measuring their titration curves, titrations were performed in the presence of formaldehyde to the amount of about 1·2 *M* in order to estimate the amounts of free primary amino-groups, by the method described by Kekwick & Cannan [1936].

Experimental. The method of titration was designed to give curves of moderate accuracy using as small an amount of solution as possible; with hydrogen electrodes it is necessary to use the rocking type of half-cell described by Clark to avoid frothing of protein solutions; cells were made on this principle [described by Ogston & Peters, 1936] with a maximum capacity of 0·2 ml. which can be used with rather less than 0·1 ml. of solution. The electrodes are small platinum plates lying parallel to the length of the cell; hydrogen flows continuously over the surface of the liquid, escaping through a trap; titrant solutions *N*/10 are added from capillary microburettes after removal of the hydrogen exit trap. Contact is made with the 3·5 *N* calomel electrode through a flexible connexion of rubber pressure tubing; closed unlubricated taps prevent any flow of liquid to or from the titration half-cell.

In order to obtain accurate and reproducible curves the following precautions were found necessary.

(1) The very small volume and titre of the solution make it especially sensitive to acid or basic impurities; trouble was experienced from the former. The hydrogen from a cylinder was washed first with ammoniacal cuprous chloride to remove oxygen, then very efficiently with 20% sulphuric acid to remove ammonia; it was then passed through two U-tubes containing "protosorb" soda-lime to remove acid spray and carbon dioxide; finally it was saturated with

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[1] Freedom Research Fellow.

water vapour at the temperature of the thermostat. As much as possible of the hydrogen tubing should be of glass, since it appears that carbon dioxide can diffuse through rubber at a rate sufficient to cause marked drifting of the potential in alkaline solutions. A further source of acid impurity was the soft-glass of the first cell: a cell was therefore constructed of pyrex glass and used only for alkaline titrations since it evolved acid for two days after exposure to acid solutions.

(2) Large errors (up to 15%) were found in volume delivery of protein solutions into the cell; it was found necessary to deliver these solutions by weight by means of a pipette made from 2 mm. capillary tubing having a long fine jet at one end and a rubber nipple at the other; this was weighed before and after discharge of solution, the error being estimated at not more than 0·2%.

(3) It was advisable to platinize the electrode afresh before every run.

Each branch of the curve was determined from two or three concordant runs with different electrodes and different portions of solution; thus each complete titration curve requires 0·4–0·6 ml. of solution which may be as dilute as 0·5%. Runs with formaldehyde were usually not repeated unless there was reason to suspect their reliability; misbehaviour of these was easy to detect. Discordant runs were very uncommon.

All measurements were made in an air thermostat at 25 ± 0·2°; potentials were measured with a Tinsley valve electrometer and slide wire potentiometer.

Materials. Horse serum proteins were used for this work since they were easily available. They were prepared by ammonium sulphate precipitations as described by Holiday [1936], except for NEG *αγ* vii which was made by dialysing whole serum; details in which the preparations differed are given in a column of the table.

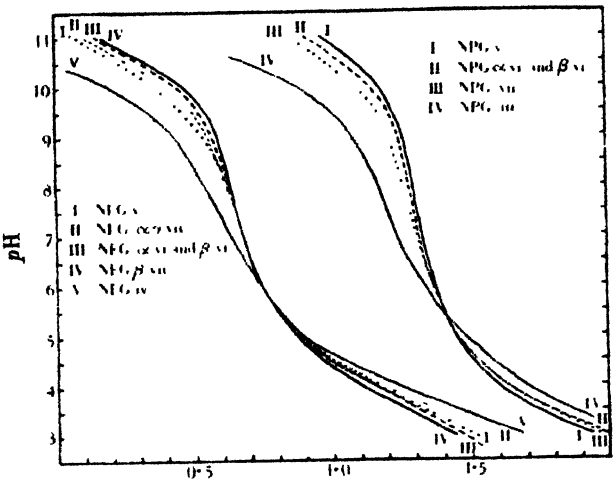
The concentrations of the protein solutions were estimated by total nitrogen determination by the micro-Kjeldahl method of Parnas & Wagner [1921], using a factor of 6·45 to convert into weight of protein. These measurements were done in parallel pairs with a control, and agreed to within 0·3%.

A strong stock solution of formaldehyde was prepared and estimated according to the directions of Levy [1933]. Formaldehyde solutions of the proteins were made up by weight in small vessels and used immediately.

Results. The results are given in the form of corrected titration curves in Fig. 1. Fig. 2 shows the actual points obtained in the titration of NPG *β* vi to show the degree of reproducibility of the method. Fig. 3 shows a specimen formaldehyde curve and the subtraction curve [*vide* Kekwick & Cannan, 1936] giving the amino-titre.

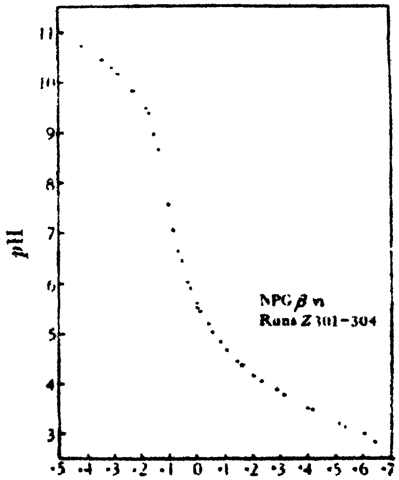
Titrations have been performed only between *p*H 3 and 11; outside these limits the correction for the water blank leads to large uncertainties (*a*) because of the increasing importance of reproducibility of the liquid junction potential at high or low *p*H; 1 mv. is the best that can be claimed with this small cell; (*b*) because of the increasing importance at high or low *p*H of the uncertainty about the values of activity coefficients in protein solutions; for lack of better information values of the activity coefficients have been assumed to be those of solutions in the absence of protein. Correction for the base-binding of formaldehyde has been applied using the formula of Levy [1934].

In Table I are given the amino-titres estimated by formaldehyde titration and the tyrosine and tryptophan contents estimated spectrographically [quoted from Holiday, 1936], expressed in milliequivalents per g. protein.



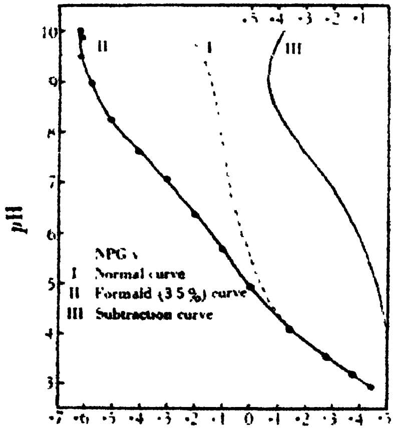
Titre; milliequivalents/g.

Fig. 1.



Titre; milliequivalents/g.

Fig. 2.



Titre; milliequivalents/g.

Fig. 3.

Table I.

Protein	% formaldehyde	Milliequivalents/g. Amino	Tyrosine	Tryptophan	Remarks
			Pseudoglobulins.		
NPG v	3·5	0·44	0·28	0·13	Three times precipitated
NPG α vi	3·9	0·47	0·29	0·13	Twice precipitated
NPG vii	3·7	0·41	0·35*	0·15*	Twice precipitated
NPG iii	3·6	0·64	0·31	0·14	Three times precipitated: dried P_2O_5
			Euglobulins.		
NEG v	3·7	0·41	0·33	0·14	Dialysis of whole globulin
NEG vi	3·8	0·44	0·37*	0·15*	
NEG β vii	3·7	0·42	0·38*	0·15*	
NEG αγ vii	3·8	0·60	0·39*	0·18*	Dialysis of serum
NEG iv	3·1	0·61	0·41	0·11	Dialysis of whole globulin; dried P_2O_5

* E. R. Holiday: Private Communication.

DISCUSSION.

Apart from the two dried proteins NPG iii and NEG iv, which show an abnormally large titre at all values of pH and abnormal amino-contents, differences in the titration curves of both pseudoglobulins and euglobulins are evident; these differences are well outside the experimental error, as is shown by the identical curves given by NPG α vi and β vi. Whilst the pseudoglobulins give the same shape of curve, differences in shape are found between the curves of the euglobulins; these latter have in general a greater titre than the pseudoglobulins. It is of interest that the two differently prepared vii euglobulins have different titration curves. The smallness of variation in the amino-figures is remarkable. They are of the same order as the highest figure obtained for globulin by Dulière [1936].

McFarlane [1935] showed that a protein cannot be dried without becoming polydisperse, and that euglobulins prepared by ammonium sulphate precipitation are more polydisperse than pseudoglobulins so prepared. If polydispersion is accompanied by any breaking up of protein molecules, new groups should become available for titrations; this might account for the large titre of the dried proteins and for the greater titre of euglobulins as compared with pseudoglobulins. But it appears that new groups appearing in euglobulins cannot be primary amino-groups; nor is there any obvious correlation between the titre in alkaline solutions and the tyrosine contents of the proteins.

The differences, at any rate of the pseudoglobulins, suggest that there may be differences in the ampholyte properties of globulins prepared from different individuals; it is interesting to note that preliminary experiments on pseudoglobulins prepared from four different specimens of the same human blood show no significant differences. These results will be published in full in due course.

SUMMARY.

1. A micro-method is described for determining the titration curves of small amounts of protein solution.

2. The titration curves of several specimens of globulins from different horse sera have been determined; further evidence is obtained of the polydispersion in solution of dried proteins and of euglobulins prepared by ammonium sulphate precipitation.

3. The results suggest that there may be differences in the properties of globulins prepared from bloods of different individuals.

The author thanks Mr E. R. Holiday for advice and encouragement.

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CCLIX. THE RELATIONSHIP BETWEEN CHEMICAL COMPOSITION AND MECHANICAL STRENGTH IN THE WOOD OF ENGLISH ASH (*FRAXINUS EXCELSIOR* LINN.).

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In the great majority of woods which have been investigated there is a strong correlation between specific gravity and certain mechanical characteristics. As a rule the higher the specific gravity the stronger the wood, although it is known that, in any single species, wide variations in strength may occur throughout a range of matched specimens of equal specific gravity. These latter variations have been ascribed [Clarke, 1935; 1936] to concomitant variations in the chemical composition of the wood cell walls which at the present time are not fully understood. Despite its obvious importance the relationship between chemical composition and strength in woody plants has hitherto received comparatively little attention. Dadswell & Hawley [1929] observed a higher cellulose content in tough white oak than in a single brash specimen of the same species, and Uno [1932] concluded that the strength of various species of bamboo increases with increasing cellulose content. The investigation by Luxford [1931] of the influence of minor components or extractives on the strength of wood will be referred to later.

The object of the present study was in the first instance to determine whether the chemical composition of ash wood varies within a single tree as well as from tree to tree in a single locality, and in the second instance to attempt to correlate chemical composition with strength figures already obtained for the same material [Armstrong, 1936].

EXPERIMENTAL.

A 2-ft. bolt was cut at the same height from the ground from each of six representative English ash trees (*Fraxinus excelsior*) obtained from Holkham, Norfolk. Each bolt was sawn into two discs measuring 15 and 9 in. in diameter respectively and the pairs of discs were marked out on the transverse surfaces exposed by the common cross-cuts into the following concentric zones.

A. Sapwood containing starch.

B, C. Intermediate zones containing small amounts of starch decreasing from B to C.

D. Heartwood containing no starch.

The 15-in. discs being reserved for mechanical tests [Armstrong, 1936] each 9-in. disc was sawn into the respective zones which, after air-drying, were separately converted into sawdust. The 80–100 mesh material was analysed according to the method of Schorger [1926] except that the digestion with 72 % sulphuric acid in the lignin determinations was carried out at 10 ± 0·5° using 12·5 ml. of acid per 2 g. sample of air-dry wood flour (Table I).

Table I.

(All results expressed as percentages by weight of oven-dry 80–100 mesh wood.)

Normal trees

Tree No. ...		1				2				3			
Zones ...		A*	B	C	D	A	B	C	D	A	B	C	D
Cold water-soluble		6·13	3·66	3·61	4·30	6·41	5·32	4·88	5·24	6·29	5·75	4·51	2·38
Hot water-soluble		7·00	4·75	5·01	5·44	7·91	6·86	5·92	5·97	9·33	8·11	7·55	6·48
1% NaOH-soluble		23·32	19·18	19·63	21·27	25·43	22·62	20·19	20·51	27·71	26·68	26·57	25·19
Alcohol-benzene (1 :2)-soluble		5·24	3·14	3·12	3·97	5·57	5·25	4·58	4·52	7·38	6·08	4·95	4·58
†Lignin content		22·53	21·74	21·81	20·39	24·80	22·31	21·47	21·34	24·19	23·14	22·61	23·01
Cellulose content		51·39	53·50	54·17	54·19	48·03	52·63	55·06	52·50	49·40	51·52	53·08	52·40
Methoxyl content		6·84	6·82	6·91	6·90	6·89	6·80	6·83	6·73	6·88	6·83	6·43	5·99
Methoxyl in lignin (i)		4·98	5·14	5·16	4·67	4·99	4·98	4·92	4·83	4·64	4·71	4·79	4·62
(i) as % of lignin		22·10	23·64	23·64	22·91	20·12	22·32	22·92	22·63	19·19	20·35	21·18	20·07
Total pentosans		22·27	22·79	23·09	23·08	25·17	23·38	21·97	23·00	25·25	24·75	26·01	24·79
Pentosans in cellulose		10·90	10·10	11·48	11·20	11·81	12·47	11·93	10·91	13·81	15·16	13·48	13·41
Pentosans not in cellulose		11·37	12·69	11·61	11·88	13·36	10·91	10·04	12·09	11·44	9·59	12·38	11·38
Specific gravity (oven-dry)	Mean for strength test pieces	0·565	0·598		0·628	0·542	0·582		0·614	0·539	0·543	0·588	0·607
‡Maximum crushing strength		6620	7760		8460	5800	6480		7280	5250	5500	6420	7560

Trees with "black-heart"

Tree No. ...		4				5				6			
Zones ...		A	B and C	D_1	D_2	A	B and C	D_1	D_2	A	B	C	D
Cold water-soluble		5·27	3·88	2·31	2·11	6·24	5·25	3·66	2·27	5·40	6·13	4·80	2·73
Hot water-soluble		6·71	5·55	4·80	4·52	8·16	7·33	6·39	4·77	6·60	7·26	6·38	4·72
1% NaOH-soluble		22·77	21·29	21·39	21·53	22·93	22·55	22·19	20·54	22·21	22·23	20·67	19·11
Alcohol-benzene (1 :2)-soluble		4·84	3·62	1·94	1·82	6·67	5·35	3·09	1·56	5·23	5·79	3·78	1·64
†Lignin content		22·48	22·62	22·40	22·27	21·49	21·38	21·53	20·86	21·33	21·90	21·37	21·49
Cellulose content		52·09	56·34	55·63	55·37	51·52	52·60	55·70	57·91	54·02	53·46	53·86	55·13
Methoxyl content		6·73	6·40	6·37	6·39	6·51	6·90	6·24	6·25	6·43	6·77	6·79	6·49
Methoxyl in lignin (i)		4·60	4·76	4·67	4·51	4·66	4·65	4·44	4·25	4·32	4·58	4·62	4·51
(i) as % of lignin		20·46	21·04	20·84	20·25	21·69	21·75	20·63	20·37	20·25	20·91	21·62	20·99
Total pentosans		23·75	23·62	23·74	23·10	23·08	23·47	23·65	23·67	22·72	23·04	22·57	22·64
Pentosans in cellulose		12·06	13·81	12·77	11·99	11·58	12·19	12·54	12·17	12·00	12·63	11·09	10·69
Pentosans not in cellulose		11·69	9·81	10·97	11·11	11·50	11·28	10·11	11·50	10·72	10·41	11·48	11·95
Specific gravity (oven-dry)	Mean for strength test pieces	0·521	0·516	0·587		0·543	0·611	0·645		0·522	0·528		0·535
‡Maximum crushing strength		5530	6180	8140		5610	6890	8500		5390	5640		6420

* A = Sapwood; B and C = Intermediate wood; D = Heartwood.

† Digestion of wood sample with 72% sulphuric acid carried out at 10°.

‡ Pounds per square inch, corrected for moisture content.

Four of the trees examined contained the heartwood discoloration known as "black-heart". This has been shown by Armstrong [1936] to have no detectable effect on the mechanical strength of the wood. The results in Table I indicate that "black-heart" does not appear to be associated with any irregularities in the relative proportions of the major components present in the trees in which it occurs but rather with some constitutional peculiarity in the extractives. This view is supported by the observation that an evaporated aqueous or alcoholic extract of "black" heartwood possessed a strong phenolic odour which was not detected in extracts obtained from normal wood. This aspect of the difference between normal and "black" heartwood could not be followed up owing to lack of available material.

DISCUSSION.

The results in Table I show that the chemical composition of ash wood is not uniform throughout the cross-section of the tree. The cellulose content exhibits a consistent increase in passing from the periphery towards the pith. The extreme differences in cellulose content between heartwood and sapwood may be as great as 7 % of the dry weight of the wood substance. It is further observed that the heartwood of ash contains less extractives and slightly less lignin than sapwood. Of the three major wood components the furfuraldehyde-yielding complexes exhibit the least fluctuations within individual trees and it is, therefore, obvious that the Cross and Bevan cellulose isolated from the heartwood contains a higher proportion of hexosans than that isolated from the intermediate zones and sapwood.

The results in Table I suggest the following two important conclusions for ash wood, which may conceivably be applicable to other hardwood species. (1) Because of the variations in composition within individual trees it would be difficult or impossible by means of a limited number of analyses carried out by current methods to differentiate chemically between trees of the same species. (2) The relatively high specific gravity and consequently the relatively high mechanical strength of heartwood is not solely due to the greater density of wood substance. An additional factor, namely the amount and composition of the Cross and Bevan cellulose, must be taken into account.

Statistical analysis of results.

The complete set of mechanical data obtained by Armstrong [1936] which correspond to the chemical data in Table I are not recorded here, but average figures are given in order to illustrate the degree of variation in compressive strength between the various zones in each tree. The discussion which follows is based upon a detailed consideration of all the mechanical and chemical data.

There are undoubtedly so many influences causing the strength of wood to vary that it is impossible by inspection of the results in Table I alone to allot effects to individual components. The method of approach used here was to fit successive regression equations involving an increasing number of variables, believed to be related to strength, the progressive improvement in the fit of the observations to the equations being taken as an indication of the importance of the term added. The considerable variation between zones was eliminated by confining consideration to variation from the mean of each zone. In a preliminary examination the best fit was provided by an equation involving specific gravity, hot water-soluble and alkali-soluble material, but since it is known that sodium hydroxide attacks all the major components of wood a search was made for an equation which would involve these latter components directly. It was found that an equation involving specific gravity and percentages by weight of cellulose, lignin and pentosans not in the cellulose was not significantly different from the first in its fit. In order to examine the specific gravity term further, each strength value was corrected to read as pounds per square inch of wood substance. These corrected values were still related to specific gravity as well as to the components already mentioned. A further improvement in fit was obtained by using the degree of dispersion (i.e. weight present in unit volume of the wood-water aggregate) of the three major components instead of their percentages by weight.

The regression equation giving the best fit to the variations within zones of the corrected strength was:

$$y-\bar{y}=102\,(m-\bar{m})+965\,(x-\bar{x})+45\,\frac{c-\bar{c}}{Vm}+69\,\frac{l-\bar{l}}{Vm}+21\,\frac{p-\bar{p}}{Vm}\ldots A,$$

where y is the maximum crushing strength in lb./sq. in. of wood substance; m is the moisture content at time of test as percentage of dry weight; x is the specific gravity of the wood block at time of test; c is the weight of cellulose per 100 g. of oven-dry (105°) wood substance; l is the weight of lignin per 100 g. of oven-dry (105°) wood substance; p is the weight of pentosans not in the cellulose per 100 g. of oven-dry (105°) wood substance; Vm is the volume occupied by a wood-water; aggregate consisting of 1 g. oven-dry wood and $m/100$ g. water. Symbols with bar indicate mean values for one zone.

No great importance can be attached to the values of the coefficients since these would no doubt be altered as a result of a more extensive investigation. It is significant, however, that each term was proved to represent an important source of variation in strength and the most important, as tested by the omission of each in turn from the equation, was found to be the cellulose term.

Finally, the observed variation in corrected strength was analysed according to the scheme set out in Table II by the methods laid down by Fisher [1935]. This type of analysis is important, firstly in dividing an observed variation into parts whose sources can be recognized (named in column 1) and secondly in enabling a test to be made of the significance of each of these sources when compared with the "residual" or "unexplained" variation (item iv).

Table II. *Analysis of variance of strength values corrected for porosity of wood.*

Variation attributed to	Sum of squares	Degrees of freedom	Mean square
(i) A regression with same coefficients as *A* but passing through the general mean	1,369,118	5	273,824
(ii) Deviation of zone means from this regression	263,308	2	131,654
(iii) Differences between zone regressions	129,889	10	12,989
(iv) Deviations from zone regressions, residual variation	333,838	84	3,974
Total	2,096,153	101	—

Co-variation of strength and chemical composition.

The fact that the percentage amounts of all the chemical components determined in Table I vary in a regular gradation across each disc does not simplify the task of explaining the strength variations, which undergo a similar regular change. The chief evidence for any one component being the cause of variations in strength would be an observed change in such component which was not reflected in changes in the other components. In the mathematical methods employed full advantage was taken of such cases where they existed. The prominence of the specific gravity term in this type of analysis is in accord with much other published work. After due allowance is made for the direct effect of bearing-area in the test piece the remaining effect must be ascribed to some of the properties which run parallel with specific gravity. Specific gravity and cellulose content both increase from the periphery towards the heart of the tree but cellulose is already a term in the equation, so that it may well be that those regions which are high in cellulose have the higher proportion of strength-giving cellulose. In this connexion it has already been noted that the Cross and Bevan cellulose of the heartwood zones (Table I) is relatively high in hexosans and low in pentosans. It is evident, however, that structural features such as ring width, proportion of summer wood etc. account for a part of the variations in strength as distinct from variations ascribed to purely chemical causes. No measurements from which it might have been possible to assess the separate effect of structural features were made in this study.

The strong relation found here between strength, specific gravity and extractives is worthy of note. It is highly probable that the so-called extractives of wood are not confined to the cell cavities, so that they may influence strength to some extent by dispersion of the cell wall substance. Luxford [1931] claims to have shown that the presence of extractives increases the compressive strength of wood, but before such a conclusion can be accepted the following point must be considered. In all the woods used by Luxford the extractives increased in amount from the sapwood towards the heartwood, whereas in the material of the present study the extractives decreased in amount in the same direction. Yet in both cases specific gravity and mechanical strength were greater towards the heartwood. It is therefore indicated that, where a measured variable is only one of a highly related group, the properties of which change regularly in passing from sapwood towards heartwood, there is a real danger in assuming cause from the existence of a single apparent relation. The results of the present study tend to weaken Luxford's [1931] conclusions, although this author made subsidiary observations of an increase in strength after the artificial addition of extractives to wood and a decrease on leaching them out. Had these latter observations been as definite as those in the main experiment, they might have made Luxford's conclusions more acceptable.

SUMMARY.

1. Analytical data are given for comparable cross-sectional discs from six English ash trees (*Fraxinus excelsior* Linn.) taken from the same site.

2. It is shown that in ash wood the percentage amount of Cross and Bevan cellulose increases in passing from the periphery towards the pith. The percentage amounts of lignin and extractives tend to decrease in the same direction while the furfuraldehyde-yielding complexes remain virtually constant in amount throughout a cross-sectional disc of any one tree.

3. The Cross and Bevan cellulose of ash heartwood contains proportionately less furfuraldehyde-yielding material and more hexosans than the corresponding component of sapwood.

4. The commercial defect known as "black-heart" in ash wood is not associated with any irregularities in the proportions of the three major components present in wood in which it occurs, but rather with a change in some minor constituent.

5. A statistical analysis is given of the effect of chemical composition on the compressive strength of ash wood, from which it is concluded that the most important component responsible for variations in crushing strength in pounds per square inch of wood substance is the Cross and Bevan cellulose. In all the material examined, specific gravity, cellulose content and strength increased in a direction from the periphery towards the pith.

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CCLX. KETOGENESIS-ANTIKETOGENESIS.

III. METABOLISM OF ALDEHYDES AND DICARBOXYLIC ACIDS.

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ALIPHATIC ALDEHYDES.

Battelli & Stern [1910] and Parnas [1910] discovered independently an enzyme system which converted aldehydes into the corresponding alcohols and acids by dismutation (the Cannizzaro reaction). Parnas found that aliphatic aldehydes incubated with liver brei anaerobically in bicarbonate buffer were dismuted quantitatively. Reichel & Berczley [1931] prepared a purified "aldehydrase"; and the dismutation of propaldehyde was found by Reichel & Wetzel [1934] and by Reichel & Köhle [1935, 1] to occur equally well under aerobic and anaerobic conditions. Reichel & Köhle [1935, 2] believe that an alcohol dehydrogenase is associated with the "aldehydrase". It is possible that the two enzymes working together aerobically could completely convert aldehyde into acid. Although the mechanism of aldehyde oxidation is not yet clear, it is of interest to examine the behaviour of aldehydes in tissue slices taking into account both the anaerobic dismutation and the ketogenic properties under aerobic conditions. This is desirable because Friedmann [1908] has observed that acetaldehyde and aldol are ketogenic.

Methods.

The general methods employed have been described earlier [Edson, 1935]. Acetoacetic acids (or β-ketonic acids) was determined by the aniline citrate procedure. Since the higher aldehydes are almost insoluble in water, it was necessary in some cases to work with suspensions. The members of the series from valeraldehyde to octaldehyde inclusive were added to the Ringer solution as fine emulsions in quantity sufficient to provide about 2 mg.

The dismutation was investigated manometrically by measuring anaerobic acid production in bicarbonate-Ringer solution at pH 7·4 and 37·5°. For such experiments it was permissible to use 30–40 mg. (dry wt.) of liver slices. Respiration and ketone body formation were measured as usual in phosphate saline [Krebs, 1933].

EXPERIMENTAL.

Anaerobic dismutation of aldehydes in surviving liver tissue.

Since the chemistry of anaerobic dismutation is fully known, the rate of reaction can be determined by following the change in one of the reactants. Though it is not possible to estimate accurately the small amounts of alcohol which would be formed in a tissue slice experiment, a precise measurement of acid production can be made. Table I shows the rate of acid formation, as measured by CO_2 evolution from bicarbonate, in rat liver slices which were shaken in presence of aliphatic aldehydes (neutral).

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Table I. *Anaerobic acid formation by rat liver slices in presence of aliphatic aldehydes.*

3 ml. bicarbonate-Ringer solution [Krebs & Henseleit, 1932]. *p*H 7·4. Duration of exp. 1 hour. Gas: 5% CO_2; 95% N_2.

Substrate	Q_{Acid}	Substrate	Q_{Acid}
Nil	2·59	*n*-Valeraldehyde, saturated	3·26
Glucose, 0·01 *M*	2·60	*iso*Valeraldehyde, saturated	3·38
Acetaldehyde, 0·01 *M*	8·80	*n*-Hexaldehyde, saturated	2·26
Propaldehyde, 0·01 *M*	7·76	*n*-Heptaldehyde, saturated	2·09
Butaldehyde, 0·01 *M*	4·15	*n*-Octaldehyde, saturated	2·54

$$Q_{\text{Acid}} = \frac{\mu\text{l } CO_2}{\text{mg. dry wt. tissue} \times \text{hours}}.$$

It will be observed that acid production occurs to a marked extent with acetaldehyde and propaldehyde; to a smaller degree with butaldehyde, *n*- and *iso*-valeraldehydes; whilst the acid production in presence of hex-, hept- and oct-aldehydes is no greater than that of the control. Acid formation decreases during ascent of the homologous series, the quotients standing in the order of the water-solubilities of the aldehydes, but there is reason to believe that tissue penetration occurs with the higher homologues, since the slices are whitened throughout at the end of an experiment.

Working with ox and pig liver brei, Parnas [1910] found a quantitative dismutation in the aldehyde series from acetaldehyde to valeraldehyde and also with heptaldehyde; rat liver slices, however, form no additional acid in presence of heptaldehyde.

Ketogenesis from aldehydes under aerobic conditions.

Since certain aliphatic aldehydes undergo dismutation in liver, it would be expected that they would also give rise to ketone bodies by secondary oxidation of fatty acids. This prediction is borne out by the experiments recorded in Table II.

Table II. *Ketone body formation from aldehydes in rat liver slices.*

Phosphate saline, *p*H 7·4, in oxygen. Duration of exp. 2 hours.

Substrate	Q_{O_2}	$Q_{\text{Acac.}}$
Nil	− 11·5	0·37
Acetaldehyde, 0·005 *M*	− 10·2	0·76
Acetaldehyde, 0·01 *M*	− 8·3	0·67
Propaldehyde, 0·005 *M*	− 10·3	0·27
Butaldehyde, 0·01 *M*	− 12·6	3·11
n-Valeraldehyde, saturated	− 13·2	2·11
*iso*Valeraldehyde, saturated	− 11·7	2·18
n-Hexaldehyde, saturated	− 12·8	4·33
n-Heptaldehyde, saturated	− 13·0	2·43
n-Octaldehyde, saturated	− 11·5	4·02

The figures of Table II show the following facts:

1. Acetaldehyde in a concentration of 0·005 *M* causes a small increase in the rate of acetoacetic acid production. Higher concentrations inhibit both respiration and ketone body formation.
2. Propaldehyde, like propionic acid, is not ketogenic.
3. Butaldehyde and its higher homologues are ketogenic and the rates of ketone body formation are approximately equal to those observed with the corresponding fatty acids, the odd-numbered members of the series giving smaller

quotients than the even-numbered members. The rate of disappearance of aldehyde is rapid, being practically complete after 2 hours. The aliphatic aldehydes react at a rate high enough to be consistent with the functions of intermediary metabolites.

It is difficult to reconcile the ketogenic actions of hex-, hept- and oct-aldehydes with their failure to exhibit dismutation anaerobically. It was thought that the ketogenesis was artificial in so far as aldehydes might be oxidized to fatty acids spontaneously. Experiments showed that no significant autoxidation occurred either in Ringer solution or in presence of boiled liver extracts during 2 hours at 37·5°. In liver slices aldehydes may be converted into ketone bodies by an alternative mechanism which does not involve dismutation.

DICARBOXYLIC ACIDS AND THEIR DERIVATIVES.

The theory of ω-oxidation has brought the dicarboxylic acids into prominence from the point of view of fat metabolism. It has long been known that some of the dicarboxylic acids such as succinic and glutaric acids are completely oxidized in the animal body [Dakin, 1921], but it was desirable to investigate their ketogenic and antiketogenic properties in liver slices of well-nourished and of starved animals. The experiments of Table III were performed in phosphate saline and β-ketonic acids were determined by the aniline citrate method. Acids were added as neutral sodium salts.

Table III. *Ketone body formation in presence of dicarboxylic acids and some of their derivatives.*

Rat liver slices.

	Well-nourished animal		Starved animal (24 hours)	
Substrate (M)	Q_{O_2}	Q_{Acac}	Q_{O_2}	Q_{Acac}
	Saturated dicarboxylic acids.			
Oxalic, 0·01	-11·0	0·58	—	—
Oxalic, 0·005	-12·3	0·98	—	—
Oxalic, 0·0025	-12·1	1·07	—	—
Control (no substrate)	-12·0	0·48	—	—
Oxalic, 0·015	—	—	- 8·9	1·63
Oxalic, 0·003	—	—	-10·9	2·17
Control	—	—	-12·1	1·97
Malonic, 0·01	-13·1	2·12	- 8·5	2·80
Malonic, 0·02	-10·2	2·88	—	—
Control	-15·1	0·60	-10·0	1·66
Succinic, 0·01	-14·6	0·26	-16·1	1·60
Control	-11·5	0·27	-10·0	1·66
Glutaric, 0·01	-12·9	0·18	-10·7	1·43
Control	-11·5	0·27	-10·0	1·66
Adipic, 0·01	-12·1	0·16	-13·6	2·31
Control	- 11·5	0·27	-10·2	2·04
Pimelic, 0·01	-13·4	0·30	-14·2	2·30
Control	-13·9	0·33	-10·2	2·04
Suberic, 0·01	-19·5	0·14	-15·1	1·96
Control	-13·9	0·33	-10·2	2·04
Azelaic, 0·01	-14·8	0·35	-13·4	1·62
Control	-13·9	0·33	-10·2	2·04
Sebacic, 0·01	-14·1	0·21	-14·1	1·43
Control	-11·5	0·27	-10·2	2·04
Thapsic, 0·1 ml. saturated solution	-10·1	0·48	-13·4	2·29
Control	-12·1	0·70	-13·7	2·55

Table III (*cont.*).

	Well-nourished animal		Starved animal (24 hours)	
Substrate (M)	Q_{O_2}	$Q_{\text{Acac.}}$	Q_{O_2}	Q_{Acac}
Unsaturated dicarboxylic acids.				
Fumaric, 0·01	−12·6	0·26	−16·4	2·81
Control	−12·1	0·27	−13·7	2·55
Maleic, 0·01	−11·9	1·07	−11·9	2·70
Control	−12·1	0·70	−13·7	2·55
Maleic, 0·01	−12·8	0·95	—	—
Maleic, 0·02	−12·4	1·09	—	—
Maleic, 0·04	−12·2	0·75	—	—
Control	−14·0	1·06	—	—
Hydroxy- and ketonic derivatives.				
Hydroxymalonic, 0·01	−13·9	3·35	−11·2	3·51
Hydroxymalonic, 0·02	−12·2	3·18	—	—
Hydroxymalonic, 0·03	−11·0	2·58	—	—
Hydroxymalonic, 0·04	− 9·5	2·07	—	—
Control	−15·1	0·60	−13·7	2·55
Malic, 0·01	−13·6	0·93	−10·5	1·63
Control	−12·1	0·70	−10·1	1·66
d-Tartaric, 0·01	−11·6	1·51	—	—
Control	−13·1	0·83	—	—
Mesoxalic, 0·01	−12·2	2·43	—	—
Control	−12·9	0·67	—	—
α-Ketoglutaric, 0·01	−11·5	0·14	−17·4	2·67
Control	−11·5	0·21	−13·7	2·55
The results with citric acid may be included with the above.				
Citric, 0·01	−13·2	0·43	−15·3	2·23
Control	−12·1	0·70	−13·7	2·55

NOTE. Concentrated solutions of aniline citrate cannot be used for the determination of acetoacetic acid in presence of mesoxalic acid since the latter decomposes slowly evolving CO_2. Instead the method of Ostern [1933] was employed, the decomposition of mesoxalic acid then being negligible. After removal of the slices 0·5 ml. 3 M acetate buffer was added to the fluid in the main compartment of the manometer vessel and to the side-bulb 0·2 ml. water and 0·05 ml. aniline. As usual the contents of the side-bulb were tipped into the main compartment after 20 min. equilibration at 25°. With quantities of acetoacetic acid corresponding to 100 μl. CO_2 pressure readings were constant in about 3·5 hours.

The following facts will be observed:

1. In general the dicarboxylic acids increase the oxygen uptake of liver slices, an effect which is most marked in starved animals. Succinic and suberic acids cause the greatest augmentation; smaller increases are observed with adipic, pimelic, azelaic and sebacic acids, and in the starved animal with α-ketoglutaric acid. Mazza [1936] has recently published similar results.
2. Certain dicarboxylic acids—malonic, hydroxymalonic, oxalic and tartaric—depress respiration when their concentrations are only 0·01 M.
3. The majority of the acids are not ketogenic in the well-nourished liver, nor are they antiketogenic in the liver of the starved animal.
4. Malonic, hydroxymalonic and mesoxalic acids are striking exceptions. Malonic acid and its hydroxy-derivative depress respiration and simultaneously produce large amounts of ketone bodies in the liver of the well-fed rat, and both are ketogenic in starved liver. Mesoxalic acid is ketogenic to a similar degree.
5. Maleic acid depresses respiration but is not ketogenic.
6. *d*-Tartaric acid is weakly ketogenic in agreement with the observations of Ohta [1912].

7. In presence of thapsic acid the respiration of well-nourished liver was diminished but ketone body formation was unaffected; larger amounts of thapsic acid strongly inhibited both respiration and ketogenesis.

8. Oxalic acid is ketogenic, particularly in low concentrations which do not inhibit respiration, but the effect is never large.

9. Citric acid is neither ketogenic nor antiketogenic under the conditions utilized.

The action of oxalate was examined in a Ca-free phosphate saline (Table IV). The results show that the ketogenic effect is not due to precipitation of Ca^{++} which occurs in ordinary Ringer-phosphate solution.

Table IV. *Ketogenic action of oxalate in rat liver slices.*

	Phosphate saline		Ca-free phosphate saline	
Substrate (*M*)	Q_{O_2}	Q_{Acac}	Q_{O_2}	Q_{Acac}
Nil	-12·8	0·36	-14·2	0·24
Oxalate, 0·01	-12·5	0·66	-11·5	0·46
Oxalate, 0·002	-13·1	0·99	-12·7	0·72
Oxalate, 0·001	-13·0	1·25	-15·5	0·73
Nil	-12·4	0·43	-13·6	0·16
Oxalate, 0·003	-13·5	1·43	-13·1	0·86
Oxalate, 0·0017	-13·6	1·56	-14·1	1·03
Oxalate, 0·0003	-13·6	0·96	-14·1	0·99

Table V. *Inhibition of malonate and hydroxymalonate ketogenesis.*

Rat liver slices.

Exp. 1.

Substrate (*M*)	Q_{O_2}	$Q_{Acac.}$
Control (no substrate)	-14·7	0·50
Malonate, 0·02	-12·4	2·77
Succinate, 0·01	-20·2	0·44
Fumarate, 0·01	-15·1	0·53
Pyruvate, 0·01	-19·1	0·98
Malonate, 0·02 + succinate, 0·01	-14·1	2·19
Malonate, 0·02 + fumarate, 0·01	-13·6	2·55
Malonate, 0·02 + pyruvate, 0·01	-17·4	2·29
Control	-12·7	0·33
Fumarate, 0·01	-14·4	0·61
Pyruvate, 0·01	-17·9	1·07
Malonate, 0·01	-12·4	1·71
Hydroxymalonate, 0·01	-11·5	2·15
Malonate, 0·01 + fumarate, 0·01	-13·0	1·36
Hydroxymalonate, 0·01 + pyruvate, 0·01	-14·6	3·38

Exp. 2. Malonate concentration constant, that of succinate varied.

	In absence of malonate		In presence of malonate, 0·007 *M*	
Substrate (*M*)	Q_{O_2}	Q_{Acac}	Q_{O_2}	Q_{Acac}
Succinate, 0·007	-15·9	0·80	-15·6	1·56
Succinate, 0·014	-18·5	0·58	-15·2	1·38
Succinate, 0·02	-19·1	0·70	-16·5	1·27
No succinate	-12·2	0·98	-14·4	2·01

Exp. 3.

Substrate (*M*)	Q_{O_2}	$Q_{Acac.}$
Control (no substrate)	-15·1	0·60
Malonate, 0·01	-11·8	2·12
Hydroxymalonate, 0·01	-10·2	2·88
Glycerol, 0·01	-15·8	0·53
Malonate, 0·01 + glycerol, 0·01	-15·8	1·17
Hydroxymalonate, 0·01 + glycerol, 0·01	-14·3	1·43

Inhibition of ketogenesis due to malonate and hydroxymalonate. It was found that the effects of malonic acid and hydroxymalonic acid were modified in the presence of certain added substrates (Table V).

Exps. 1 and 2 show that there is a small but distinct inhibition of malonate ketogenesis in presence of succinate, fumarate and pyruvate; the value of Q_{Acac} falls as the oxygen consumption rises, the effect being more marked with higher concentrations of succinate. Pyruvate has no significant influence on ketogenesis caused by hydroxymalonate. Exp. 3 shows a strong inhibition due to glycerol; oxygen uptake is increased and ketone body formation is reduced in both cases.

DISCUSSION.

Consideration of all the facts now available leads to the conclusion that there are two classes of ketogenic substances. The first and larger class consists of substances which are converted into ketone bodies during metabolism: it includes fatty acids, some of their derivatives and the amino-acids leucine, tyrosine and phenylalanine. The existence of this group was established chiefly by the classical work of Embden and of Friedmann. The second class is composed of substances which are not converted into ketone bodies: they influence the metabolism anticatalytically, causing an accumulation of ketone bodies. Certain dicarboxylic acids, e.g. malonic acid, are representatives of this class, and ammonia should probably be placed in the same category. Jowett & Quastel [1935] have obtained evidence which caused them to reject the view that malonic acid could be transformed into acetoacetic acid via acetic acid. The conversion of the other ketogenic dicarboxylic acids (hydroxymalonic, mesoxalic, tartaric and oxalic) into ketone bodies also appears to be unlikely.

The ketogenic effect of malonic acid is now well established. It was first observed by Momose [1914] in liver perfusion experiments. Szent-Györgyi *et al.* [1935] showed that ketone bodies were excreted by a rabbit which had been injected intravenously with sodium malonate. In the absence of other substrates malonate also caused a production of acetoacetic acid in kidney slices, an effect which was inhibited by fumarate. Jowett & Quastel [1935] have studied the malonate effect in detail: they believe that malonic acid inhibits the normal breakdown of acetoacetic acid formed spontaneously from fatty acids already present in liver. Further, Quastel & Wheatley [1935] have shown that malonate prevents aerobic, but not anaerobic, disappearance of acetoacetic acid added to kidney and liver slices.

An alternative hypothesis dependent on substrate competition may be offered. Krebs [1936] has recently discovered a mechanism whereby succinic acid is formed from pyruvic acid in animal tissues. Moreover, malonate is known to be a specific inhibitor of succinic dehydrogenase [Thunberg, 1920; 1933; Quastel & Whetham, 1925; Quastel & Wheatley, 1931]. If the oxidation of carbohydrate be inhibited by malonate at the succinic acid stage, it is possible that fatty acids will be oxidized preferentially. This would account for the great increase in the spontaneous ketogenesis which is observed in presence of malonate. Similar causes may operate in other cases, e.g. (1) hydroxymalonate is known to be a strong inhibitor of lactic dehydrogenase [Quastel & Wooldridge, 1928]; (2) oxalate is a weak inhibitor of muscle succinic dehydrogenase [Thunberg 1920; 1933]; oxalic and tartaric acids inhibit the lactic dehydrogenase of toluene-treated *B. coli* [Quastel & Wooldridge, 1928]. Thus the actions of the ketogenic dicarboxylic acids may be ascribed to their specific anticatalytic properties, which hinder carbohydrate oxidation and thereby favour fatty acids in the competition for available oxygen. Evidence relating to substrate competition in liver will be presented in the next paper of this series.

SUMMARY.

1. Anaerobically there is an acid production in rat liver slices in presence of acetaldehyde, propaldehyde, butaldehyde, *n*-valeraldehyde and *iso*valeraldehyde but not in presence of hex-, hept- and oct-aldehydes. The acid is probably formed by dismutation.

2. Under aerobic conditions the above aldehydes, with the exception of propaldehyde, are ketogenic in liver. The ketone body formation is of the same order as that found with the corresponding fatty acids.

3. The metabolism of dicarboxylic acids and their derivatives has been investigated in rat liver slices. In general there is increased oxygen consumption and the acids are neither ketogenic in well-nourished liver nor antiketogenic in the liver of the starved animal. Notable exceptions are malonic, hydroxymalonic, mesoxalic, tartaric and oxalic acids. The first three are strongly ketogenic; tartaric and oxalic acids weakly ketogenic.

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CCLXI. KETOGENESIS-ANTIKETOGENESIS.

IV. SUBSTRATE COMPETITION IN LIVER.

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Krebs [1935] has shown that the deamination of *l*-amino-acids in surviving kidney tissue is inhibited by readily oxidizable substrates such as lactate and pyruvate. Although less amino-acid is metabolized, the oxygen consumption remains constant, indicating that the second substrate is oxidized instead of the amino-acid. These experiments demonstrate clearly that when kidney tissue is offered several substrates there is competition for the oxygen available. Krebs also observed substrate competition in respiring yeast cells.

The experiments of Embden & Wirth [1910] suggest that the same phenomenon can take place in liver. Perfusing the liver with various combinations of substrates, they found that the usual ketone body formation due to *iso*valeric acid was greatly inhibited by *n*-valeric acid. In a similar way *n*-valeric acid partially inhibited ketogenesis caused by *n*-hexanoic acid and by tyrosine; whilst α-amino-*n*-hexanoic acid, which itself is non-ketogenic, prevented acetoacetic acid production from leucine. When glutaric acid and glucose were separately opposed to ketogenic fatty acids, no antiketogenic effects were observed, and the position of glycerol was uncertain. Quastel & Wheatley [1933], using tissue slices, found that propionic acid, but not glucose or lactic acid, inhibited the formation of acetoacetic acid from butyric acid.

Macallum [1930] enunciated a general theory of antiketogenesis which in effect is a theory of substrate competition, although it was not formulated in those terms. The experiments of Krebs [1935] prove the reality of substrate competition, and so it became desirable to investigate this process in liver: first, by opposing different substrates to the spontaneous ketogenesis of starved liver, and secondly by allowing a wide range of substrates to compete with added fatty acids for the oxidizing enzymes of the liver of a well-nourished animal.

Methods.

Respiration and ketone body formation were measured by the methods already described [Edson, 1935]. β-Ketonic acids were determined by the aniline citrate method except in a few cases which were referred to the Van Slyke procedure. Rat tissue slices were used throughout, particular care being taken that the animals were in a good state of nutrition. Fresh food was offered to them 3 hours before they were killed; starved animals were deprived of food for 24 hours.

The competing substrates were generally present in equimolecular concentrations, 0·01 *M*, but sometimes the effect of unequal competition was investigated. The medium was Krebs's phosphate saline [1933].

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EXPERIMENTAL.

Antiketogenesis in starved liver.

Facts relating to the antiketogenic properties of amino-acids and dicarboxylic acids have been recorded in communications II and III of this series. Additional results obtained with other substrates are presented in the following tables.

Table 1. *Carbohydrates and their derivatives.*

Liver slices of starved rat.

Substrate (*M*)	Q_{O_2}	Q_{AcAc}
1. *Hexoses.*		
Nil	− 9·4	2·43
Glucose, 0·02	− 13·9	1·20
Fructose, 0·02	− 12·4	1·19
Mannose, 0·02	− 12·6	1·42
Galactose, 0·02	− 12·8	1·17
Nil	− 13·6	2·88
Glucose, 0·01	− 14·0	2·53
Glucose, 0·02	− 15·0	2·21
Glucose, 0·60	− 8·7	0·99
Nil	− 12·1	2·44
Hexosediphosphate, 0·01	− 13·8	1·53
2. *Pentoses.*		
Nil	− 12·9	2·41
Arabinose, 0·01	− 11·0	1·80
Xylose, 0·01	− 13·2	2·20
Nil	− 12·0	1·60
Rhamnose, 0·01	− 10·4	1·43
3. *Sucrose.*		
Nil	− 12·0	1·60
Sucrose, 0·01	− 12·2	1·62
Invert sugar (sucrose, 0·01 + 0·1 ml. 1% invertase in presence of liver slices)	− 15·2	0·86
0·1 ml. invertase (1% sol.)	− 12·4	1·70
4. *Glycogen.*		
Nil	− 12·7	2·43
Glycogen, 0·2 ml. 6% sol.	− 12·5	2·35
5. *Derivatives.*		
Nil	− 13·6	2·88
dl-Glyceraldehyde, 0·01	− 11·1	0·96
Nil	− 9·7	2·43
Dihydroxyacetone, 0·01	− 13·7	1·36
Nil	− 10·5	1·43
Lactate, 0·01 (*dl*)	− 16·2	0·76
Pyruvate, 0·01	− 15·3	0·97
Nil	− 12·1	2·21
α-Glycerophosphate, 0·01	− 13·3	2·26
Nil	− 12·1	2·44
β-Phosphoglycerate, 0·01	− 11·1	1·35

Table II. *Alcohols.*

Liver slices of starved rats.

Substrate (M)	Q_{O_2}	$Q_{\text{Acac.}}$
Nil	−12·0	1·60
Ethyl alcohol, 0·005	−11·0	1·46
Methyl alcohol, 0·005	−12·3	1·48
Methyl alcohol, 0·0025	−13·1	1·68
Ethylene glycol, 0·01	−12·6	1·86
Nil	−9·7	2·43
Glycerol, 0·01	−11·7	0·63
Nil	−13·6	2·88
Glycerol, 0·01	−16·7	1·19
Glycerol, 0·02	−15·4	0·84
Nil	−12·9	2·41
Erythritol, 0·01	−11·6	1·88
Adonitol, 0·01	−13·2	1·90
Dulcitol, 0·01	−11·6	2·21
Mannitol, 0·01	−12·3	1·99
Sorbitol, 0·01	−15·8	0·59
Nil	−13·6	2·88
Sorbitol, 0·01	−17·0	0·85

Table III. *Some miscellaneous results.*

Liver slices of starved rats.

Substrate (M)	Q_{O_2}	$Q_{\text{Acac.}}$
Nil	−13·6	2·88
Ornithine, 0·01	−13·1	1·96
Arginine, 0·01	−12·6	1·77
Glutaric acid, 0·01	−12·9	2·26

Note. The experiments with the higher alcohols were performed on receipt of the information that Dr B. Mendel had discovered the antiketogenic effect of sorbitol (private communication to Dr H. A. Krebs). The action of glycerol had been found previously.

The data of Tables I, II and III show the following facts with regard to the ketogenesis of starvation.

1. Glycerol, sorbitol and glyceraldehyde are strongly antiketogenic, the inhibition being 70% or over. There is increased oxygen uptake in presence of sorbitol and glycerol.
2. The other alcohols have no effect or inhibit weakly.
3. Glucose is no more antiketogenic that the other hexoses, whose influence is seen to be much smaller than that of glycerol. The antiketogenic action of glucose increases with concentration, but in order to produce an effect as great as that characteristic of 0·01 M glycerol the glucose concentration must be raised to 0·6 M, which is sufficient to depress respiration.
4. Lactate and pyruvate reduce ketone body formation by nearly 50%.
5. Added glycogen is not antiketogenic.

It appears that almost any oxidizable substrate will inhibit the spontaneous ketogenesis of starved liver to a greater or less degree.

Substrate competition in the liver of the well-fed animal.

Many experiments were performed in which substrates were allowed to compete with added fatty acids in the normal glycogen-rich liver. The results of typical experiments are recorded below. Fatty acids were added as sodium salts.

Table IV.

A. *Glucose in competition with* n-*hexanoic acid.*

Substrate (M)	Q_{O_2}	Q_{acac}
n-Hexanoic acid, 0·01	−14·3	4·91
,, ,, +glucose, 0·005	−13·8	4·97
,, ,, +glucose, 0·008	−14·1	5·21
,, ,, +glucose, 0·01	−13·9	4·42
,, ,, +glucose, 0·02	−14·3	4·63
,, ,, +glucose, 0·05	−13·7	3·86
,, ,, +glucose, 0·10	−12·4	3·20
B. *Sodium lactate in competition with even-numbered fatty acids.*		
Nil	−12·8	0·22
dl-Lactate, 0·01	−15·4	0·29
n-Butyric acid, 0·01	−17·7	3·30
,, ,, +lactate, 0·01	−18·3	2·11
n-Hexanoic acid, 0·01	−14·7	5·18
,, ,, +lactate, 0·01	−18·1	3·11
,, ,, +lactate, 0·02	−19·0	2·92
n-Octanoic acid, 0·005	−12·9	4·10
,, ,, +lactate, 0·01	−15·8	4·40
C. *Sodium pyruvate in competition with even-numbered fatty acids.*		
Nil	−12·9	0·28
Pyruvate, 0·01	−15·4	0·41
n-Butyric acid, 0·01	−16·5	2·61
,, ,, +pyruvate, 0·01	−19·5	1·59
n-Hexanoic acid, 0·01	−15·6	4·29
,, ,, +pyruvate, 0·01	−20·4	3·07
n-Octanoic acid, 0·005	−12·3	2·61
,, ,, +pyruvate, 0·01	−17·0	2·98
D. *Amino-acids in competition with even-numbered fatty acids.*		
Nil	−12·3	0·42
dl-Alanine, 0·01	−12·3	0·29
Glycine, 0·01	−11·6	0·54
n-Butyric acid, 0·01	−13·9	2·16
,, ,, +alanine, 0·01	−14·0	2·78
,, ,, +glycine, 0·01	−13·5	2·15
n-Hexanoic acid, 0·01	−14·5	3·25
,, ,, +alanine, 0·01	−15·0	3·98
,, ,, +glycine, 0·01	−14·1	3·61
Nil	−12·7	0·82
l-Tyrosine, saturated	−13·0	1·32
l-Cysteine, 0·01	−16·6	0·89
n-Butyric acid, 0·01	−15·1	3·81
,, ,, +tyrosine, saturated	−20·2	5·27
,, ,, +cysteine, 0·01	−15·2	2·75
n-Hexanoic acid, 0·01	−17·4	6·90
,, ,, +tyrosine, saturated	−16·9	6·02
,, ,, +cysteine, 0·01	−22·5	6·20
Nil	−12·2	0·11
d-Arginine, 0·01	−11·5	0·19
d-Valine, 0·01	−11·7	0·21
n-Butyric acid, 0·01	−16·8	1·98
,, ,, +arginine, 0·01	−16·3	1·66
,, ,, +valine, 0·01	−17·4	2·33
n-Hexanoic acid, 0·01	−14·4	2·63
,, ,, +arginine, 0·01	−15·1	2·13
,, ,, +valine, 0·01	−14·4	2·79
Nil	−10·2	0·33
d-Ornithine, 0·01	−12·3	0·23
n-Butyric acid, 0·01	−13·4	2·76
,, ,, +ornithine, 0·01	−12·7	2·51
n-exanoic acid, 0·01	−15·4	5·19
,, ,, +ornithine, 0·01	−13·7	3·84

Table IV (*cont.*).

Substrate (*M*)	Q_{O_2}	$Q_{\text{Acac.}}$
E. *Dicarboxylic acids in competition with even-numbered fatty acids.*		
Nil	−10·2	0·33
Succinate, 0·01	−17·9	0·44
n-Butyric acid, 0·01 +	−13·4	2·76
,, ,, + succinate, 0·01	−18·2	2·32
n-Hexanoic acid, 0·01	−15·4	5·19
,, ,, + succinate, 0·01	−19·5	4·66
Nil	−11·9	0·52
Glutarate, 0·01	−14·2	0·61
Sebacate, 0·01	−14·5	0·21
n-Butyric acid, 0·01	−13·7	2·25
,, ,, + glutarate, 0·01	−16·1	3·32
,, ,, + sebacate, 0·01	−14·1	1·78
n-Hexanoic acid, 0·01	−13·3	3·76
,, ,, + glutarate, 0·01	−15·3	5·10
,, ,, + sebacate, 0·01	−14·5	4·51
F. *Glycerol, sorbitol and glyceraldehyde in competition with even-numbered fatty acids.*		
Nil	−10·2	0·41
n-Butyric acid, 0·01	−13·0	2·35
,, ,, + glycerol, 0·01	−14·3	1·72
,, ,, + glycerol, 0·02	−14·3	1·70
n-Hexanoic acid, 0·01	−15·0	3·92
,, ,, + glycerol, 0·01	−15·0	2·80
n-Octanoic acid, 0·005	−15·0	3·93
,, ,, + glycerol, 0·01	−14·3	2·97
Nil	−11·3	0·36
n-Butyric acid, 0·01	−17·0	2·96
,, ,, + sorbitol, 0·01	−18·2	1·98
n-Hexanoic acid, 0·01	−17·2	4·48
,, ,, + sorbitol, 0·01	−17·7	3·78
n-Octanoic acid, 0·005	−14·8	4·20
,, ,, + sorbitol, 0·01	−15·0	4·88
Nil	−10·7	0·28
n-Butyric acid, 0·01	−14·6	2·48
,, ,, + *dl*-glyceraldehyde, 0·01	−10·9	1·84
n-Hexanoic acid, 0·01	−16·6	4·91
,, ,, + *dl*-glyceraldehyde, 0·01	−13·1	2·10
G. *Odd-numbered in competition with even-numbered fatty acids.*		
Nil	−11·6	0·26
n-Butyric acid, 0·01	−13·9	1·49
,, ,, + formate, 0·01	−13·6	1·75
n-Hexanoic acid, 0·01	−13·5	2·78
,, ,, + formate, 0·01	−14·9	2·83
n-Octanoic acid, 0·005	−13·7	2·92
,, ,, + formate, 0·01	−12·7	2·59
Formate, 0·01	−11·3	0·25
Nil	−9·6	0·21
n-Butyric acid, 0·01	−12·6	1·64
,, ,, + propionate, 0·01	−12·8	0·91
,, ,, + propionate, 0·02	−12·3	1·01
n-Hexanoic acid, 0·01	−15·7	3·63
,, ,, + propionate, 0·01	−15·4	2·59
Propionate, 0·01	−11·1	0·30
Nil	−13·7	0·20
n-Butyric acid, 0·01	−15·6	2·03
,, ,, + *n*-valerate, 0·01	−14·3	1·35
n-Hexanoic acid, 0·01	−16·9	3·30
,, ,, + *n*-valerate, 0·01	−18·3	1·71
n-Octanoic acid, 0·005	−13·1	2·45
,, ,, + *n*-valerate, 0·01	−16·6	2·33
n-Valerate, 0·01	−13·4	0·94

Table IV G (*cont.*).

Substrate (*M*)	Q_{O_2}	$Q_{\text{Acac.}}$
Nil	-12·6	0·40
n-Butyric acid, 0·01	-15·9	2·23
,, ,, + *n*-heptanoate, 0·005	-15·0	1·97
n-Hexanoic acid, 0·01	-17·4	4·20
,, ,, + *n*-heptanoate, 0·005	-21·4	2·59
n-Octanoic acid, 0·005	12·0	2·80
,, ,, + *n*-heptanoate, 0·005	-14·3	2·70
n-Heptanoate, 0·005	-16·9	1·48
Nil	-12·7	0·23
n-Butyric acid, 0·01	-16·5	2·76
,, ,, + *n*-nonanoate, 0·0025	-16·1	2·15
n-Hexanoic acid, 0·01	-14·6	3·77
,, ,, + *n*-nonanoate, 0·0025	-17·6	2·84
n-Octanoic acid, 0·005	-13·1	3·65
,, ,, + *n*-nonanoate, 0·0025	-15·8	3·88
n-Nonanoate, 0·0025	-14·6	1·47

H. *Even-numbered fatty acids competing with one another.*

Nil	-12·9	0·85
n-Butyric acid, 0·01	-15·8	3·65
n-Hexanoic acid, 0·01	-14·5	5·20
n-Octanoic acid, 0·005	-13·5	3·87
Acetic acid, 0·01	-15·9	1·53
n-Butyric acid, 0·01 + *n*-hexanoic acid, 0·01	-15·7	5·76
n-Butyric acid, 0·01 + *n*-octanoic acid, 0·005	-13·1	4·56
n-Hexanoic acid, 0·01 + *n*-octanoic acid, 0·005	-13·4	4·77
n-Butyric acid, 0·01 + acetic acid, 0·01	-16·7	3·88
n-Hexanoic acid, 0·01 + acetic acid, 0·01	-15·3	5·40

The figures of the above table demonstrate the following facts.

1. Many oxidizable substances inhibit the formation of β-ketonic acids from fatty acids which have been added to liver slices. The inhibition is variable in degree, and it is usually produced without lowering of the oxygen uptake. Ketogenesis from *n*-octanoic acid appears to be difficult to inhibit.

2. Glucose, glycerol, sorbitol and glyceraldehyde behave as in the starved liver, but in presence of 0·01 *M* fatty acids the percentage inhibitions are smaller.

3. Lactate and pyruvate also inhibit, the oxygen consumption then being raised above that due to fatty acid alone.

4. The amino-acids cysteine, valine and alanine have little effect, but arginine and ornithine diminish ketogenesis.

5. The dicarboxylic acids—succinic, glutaric, sebacic—have no regular influence. Succinic acid inhibits slightly; glutaric acid appears to increase ketogenesis in presence of added fatty acid.

6. *Odd-numbered fatty acids.* Formic acid is indifferent towards the process of ketogenesis, but propionic acid inhibits it to a marked extent. Since these two substrates are non-ketogenic their behaviour is not surprising. Contrary to expectation, and in spite of their own capacity to yield ketone bodies, the higher homologues—*n*-valeric, *n*-heptanoic and *n*-nonanoic acids—inhibited the formation of β-ketonic acids from butyric and *n*-hexanoic acids; but they showed no inhibitory effect in the case of *n*-octanoic acid. The antiketogenic action was accompanied, not by diminished, but by increased oxygen uptake.

7. When two even-numbered fatty acids were present together there was not a summation of the separate ketogenic effects. Instead $Q_{\text{Acac.}}$ assumed a maximum value which was a little in excess of the quotient for the more strongly ketogenic member of the pair.

dl-*Glyceraldehyde*. The results obtained with glyceraldehyde must be interpreted with caution, since it was found that there is a considerable disappearance of acetoacetic acid when it is shaken with glyceraldehyde for 2 hours at 37·5° in the absence of tissue slices. Moreover, the effect of glyceraldehyde is not increased by the presence of slices (liver or kidney). The interaction of acetoacetic acid and glyceraldehyde resembles an effect described by Henze [1930; 1931; Henze & Müller, 1930] who found that acetoacetic acid reacted with methylglyoxal *in vitro* to give "ketol".

DISCUSSION.

These results afford evidence in favour of the hypothesis that the rate of production of ketone bodies in liver is the outcome of competition between fatty acids and other oxidizable substrates, and since the liver is the chief site of ketone body formation, substrate competition becomes an important factor in regulating the general ketogenic-antiketogenic balance. If oxidizable substrates such as glucose, sorbitol and lactate were able to compete with fatty acids for available oxygen, and if this were dependent on local substrate concentration, then fatty acid oxidation might be inhibited in the way that has been observed. According to this view the antiketogenic power of carbohydrates and their metabolic derivatives depends on "sparing" of fatty acids.

Fatty acids are known to be strongly adsorbed. Should this occur in liver, it would help to explain the apparent selective oxidation of fatty acids. The oxidation of the higher homologue, *n*-octanoic acid, is scarcely affected by the presence of competitors, a fact which could be predicted if fatty acids are adsorbed by enzyme systems according to Traube's rule.

The effect of the odd-numbered fatty acids is noteworthy. If an even-numbered and an odd-numbered acid compete with each other on equal terms, inhibition may be expected, because the rates of ketone body formation are approximately in the ratio, odd : even :: 1 : 3. The observed $Q_{\text{Acac.}}$ of a competing pair is roughly the mean of the separate values obtained when only one acid is present.

A theory of substrate competition would require that the antiketogenic action should increase with the concentration of the antiketogenic substance, and that it should occur without diminution of oxygen uptake. In the case of glucose this is true, but it is not equally clear with other substrates. Regarding oxygen consumption, however, the requirements of the theory appear to be satisfied in all cases. The anticatalytic effects of malonate and other ketogenic dicarboxylic acids supply additional evidence in support of a competition theory.

Apart from substrate competition there may be other mechanisms controlling ketogenesis; for instance the powerful antiketogenic effects of glycerol and sorbitol may require some special explanation.

SUMMARY.

1. Antiketogenesis has been studied in liver slices of starved rats and in slices of well-nourished livers in presence of added fatty acids.

2. The evidence leads to the conclusion that fatty acids compete with other oxidizable substrates for the oxidizing systems of the liver. Carbohydrates and their derivatives, e.g. lactate, pyruvate and dihydroxyacetone, alcohols and certain amino-acids are antiketogenic competitors. The fatty acids also compete amongst themselves.

3. Glycerol and sorbitol are the most powerful of the antiketogenic substances that have been examined.

4. Substrate competition is considered to be an important factor in the regulation of hepatic ketogenesis.

I am greatly indebted to Dr H. A. Krebs who suggested the hypothesis on which this work was based and gave me much helpful advice.

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CCLXII. AEROBIC BREAKDOWN OF GLUCOSE BY *BACT. SUBOXYDANS.*

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BACT. SUBOXYDANS, an acetic acid organism isolated by Kluyver & de Leeuw [1924], is described by them as "characterized by its slight intensity of oxidation which leads to an accumulation of intermediary products". Normally, in aerated cultures grown in suitable liquid media, *Bact. suboxydans* converts glucose into gluconic acid and sometimes further into 5-ketogluconic acid. In this respect it differs considerably from related organisms such as *Bact. aceti*, *Bact. rancens* and *Bact. xylinum*, which are capable of oxidizing both glucose and gluconic acid to carbon dioxide and water. In the work to be described it is shown that the property of restricted oxidation so characteristic of *Bact. suboxydans* in aerobic conditions can be changed into one of strong oxidative ability similar to that of the three organisms mentioned. Moreover, the two types of culture possessing these different oxidative properties are convertible one into the other by a simple modification of the medium on which the organism is grown.

The present investigation shows that two very different types of suspension are obtained when *Bact. suboxydans* is grown on maize wort agar or on maize wort agar to which 2% chalk has been added. Suspensions derived from the medium without chalk are dirty white in colour and are capable of oxidizing glucose to gluconic acid only. They give no oxygen uptake or evolution of carbon dioxide with gluconic acid. Suspensions from cultures grown on the medium containing chalk are pink and give a very much larger and more rapid oxygen uptake with glucose than the other type of suspension, as well as a considerable evolution of carbon dioxide. Moreover, with gluconic acid a rapid oxygen uptake and evolution of carbon dioxide are obtained. It is concluded from the experimental figures that with this type of suspension glucose is first oxidized to gluconic acid, which is then broken down with evolution of carbon dioxide equal to the volume of oxygen absorbed. Some of the glucose or gluconic acid is probably oxidized to a keto- or aldehydo-gluconic acid. It is of interest in connexion with the large increase in oxygen uptake resulting from growth of *Bact. suboxydans* on the medium containing chalk, that in a recent publication Cozic [1936] states that a considerable increase in the rates of oxygen absorption by suspensions of *Bact. rancens* and *Bact. pasteurianum* was observed when chalk was added to the growth medium. It should also be mentioned that Simon [1930] showed that *Bact. suboxydans*, together with *Bact. pasteurianum* and *Bact. ascendens*, can act anaerobically on glucose to give a true alcoholic fermentation, but he states that this does not occur in the presence of oxygen.

The striking difference in oxidative powers and of ability to break down the glucose molecule of the two types of suspensions implies that the enzymic system possessed by suspensions of *Bact. suboxydans* grown on maize wort agar containing chalk is profoundly different in function from that possessed by suspensions derived from cultures grown on maize wort agar without chalk. It is very probable that the difference is due directly to the neutralization by the chalk of the acid formed by the organism during growth on the medium. Without chalk the strongly acid conditions which develop are probably unfavourable for the formation in the enzyme system of the organism of the specific active group responsible for the breakdown of the glucose or gluconic acid molecule. It is also possible that the pink pigment present in the more active suspension is partly responsible for the increased activity by acting as carrier between the activated substrate molecules and molecular oxygen, in much the same way as other intracellular pigments such as the well-known yellow enzyme and the blue pigment of *Bact. pyocyaneum.*

An attempt has been made to link this difference in properties with the intensity of phosphatase activity in the suspensions.

EXPERIMENTAL.

Bacterial suspensions and preparations. The culture of *Bact. suboxydans* used in these experiments was obtained from the National Collection of Type Cultures. The history of the different subcultures previous to use in the experiments described had varied considerably. For most of the time all cultures were on maize wort agar, but at some period they had been on beer agar, ordinary wort agar, yeast-glycerol liquid medium, bouillon-agar and maize wort agar with chalk. The media employed for growth in the experiments to be described consisted of (*a*) maize wort agar, (*b*) maize wort agar with 2 % chalk, contained in flat 16-oz. bottles. In order to incorporate the chalk throughout the agar it was essential to shake the bottles just before solidification. A thin saline suspension of the organism grown on the same medium was used for inoculation and the bottles were incubated at 30° for 4 days. The growth was removed from the agar surface by adding sterile physiological saline and rubbing with a sterile glass rod. The washings thus obtained from the maize wort agar without chalk were very acid and, to remove this acidity it was necessary, after filtering through sterile glass wool to remove small lumps of agar, to centrifuge and wash three or four times before finally suspending in saline. The organisms from the chalk medium were much more difficult to centrifuge and gave a definitely pink suspension, in contrast to the dirty white of the other suspensions. The suspensions were aerated for 1 hour and kept at 0°. The dry weights of the suspensions from the maize wort agar without chalk were usually 10–15 mg./ml. and those from the chalk medium 20–25 mg./ml. Dilution within a wide range made no appreciable difference; e.g. a suspension from the chalk medium, with dry weight 23 mg./ml., gave almost identical values for oxygen uptake and evolution of carbon dioxide with glucose when diluted to three times its volume.

The air-dried preparations were made by centrifuging a portion of the suspensions, drying the sediment in a vacuum desiccator and grinding to a fine powder.

The acetone preparations were made from the suspensions as described by Müller [1931].

Oxygen absorption and evolution of carbon dioxide of suspensions of Bact. suboxydans *with glucose and calcium gluconate.* These were measured by means of the Barcroft differential manometer, the usual technique being followed [Dixon, 1934]. The volumes of the two flasks in each apparatus were so adjusted by means of glass beads that their difference was less than 100 μl. The carbon dioxide output was measured by the direct method. The left flask of the manometer contained 1 ml. suspension and 2 ml. buffer, the right flask 1 ml. suspension, 1·7 ml. buffer and 0·3 ml. substrate contained in a Keilin tube during equilibration, after which it was mixed with the other contents. Either phosphate or phthalate buffers of *p*H 5·5 were used, preliminary experiments having shown that 5·5 is the optimum *p*H for oxygen absorption by *Bact. suboxydans* with glucose as substrate. 0·1 *M* glucose was used. Theoretically 0·3 ml. of 0·1 *M* glucose takes up 334 μl. of oxygen at N.T.P. for each atom of oxygen absorbed by the glucose molecule. A temperature of 30° was used throughout.

Suspensions of Bact. suboxydans *grown on maize wort agar (no chalk).*

Fig. 1 shows an oxygen uptake-time curve typical of many which have been obtained at different times with glucose as substrate. The oxygen uptake was comparatively rapid at the beginning and slowly decreased until it ceased altogether at a value of 320–340 μl. oxygen, i.e. at a value equivalent to the absorption of one atom of oxygen by each molecule of glucose represented by 0·3 ml. substrate. No carbon dioxide was evolved. The increase in acidity of the right-hand flask containing suspension and glucose almost invariably amounted to 1·5 ml. of 0·02N NaOH, which is equivalent to the gluconic acid obtainable from 0·3 ml. of 0·1M glucose. The absorption of the equivalent of one atom of oxygen and a simultaneous appearance of an equivalent acidity points very strongly to the formation of gluconic acid. The only other possible position for a carboxyl group in the intact glucose molecule would be at the other end of the chain and if this were formed it would require the absorption of the equivalent of two atoms of oxygen.

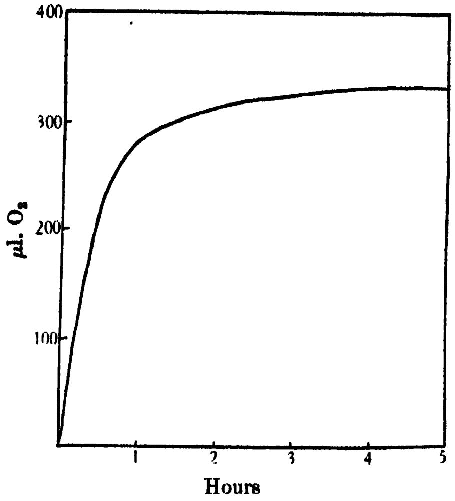

Fig. 1. Oxygen uptake, with 0·3 ml. of 0·1 M glucose as substrate, of suspension of *Bact. suboxydans* from growth on maize wort agar without chalk. Theoretical O_2 uptake for complete oxidation to gluconic acid is 334 μl. Temp. 30°. pH, 5·5.

There was no oxygen uptake or evolution of carbon dioxide with 0·05M calcium gluconate at pH 5·5.

It was thought possible that the thorough washing which this type of suspension required to remove all acid might also have removed some active agent of the enzymic system. Accordingly a portion of a suspension was centrifuged, the supernatant liquid removed and the suspension made up again with some of the neutralized supernatant liquid of the first washing. The new suspension was examined for oxygen absorption and evolution of carbon dioxide in presence of glucose in the Barcroft differential manometer and was found to give similar results to the above. It was concluded that washing had had no de-activating effect.

Suspensions of Bact. suboxydans *grown on maize wort agar containing 2 %₀ chalk.*

Fig. 2 shows a typical example of an oxygen uptake-time and CO_2 output-time curve obtained with this type of suspension using 0·3 ml. of 0·1 *M* glucose as substrate. The initial uptake of oxygen is much more rapid than that obtained with the type of suspension derived from *Bact. suboxydans* grown without chalk. The rate of oxygen absorption slowly diminishes, especially after the equivalent of one atom of oxygen has been taken up. At first there is only very little, if any, evolution of carbon dioxide, but this rapidly increases. After a time the evolution of carbon dioxide becomes equal in volume to the oxygen absorbed. The difference in volume between the total oxygen uptake and the carbon dioxide evolved represents the absorption of oxygen without any formation of carbon dioxide, that is, some simple oxidation process. In some cases this difference in volume becomes stabilized at about 330 μl. which is approximately equivalent to one atom of oxygen for each molecule of glucose present. This excess of total oxygen uptake over carbon dioxide output is in all probability due to the formation of gluconic acid, which is oxidized as soon as produced giving an evolution of carbon dioxide equal to the oxygen absorbed.

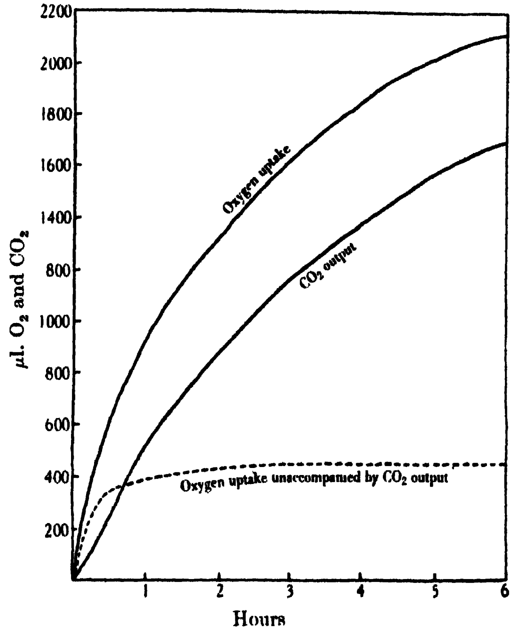

Fig. 2.

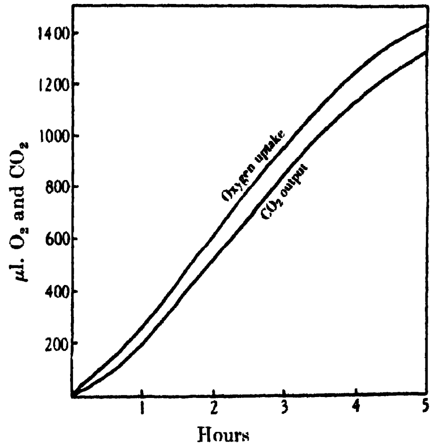

Fig. 3.

Fig. 2. Oxygen uptake and CO_2 output, with 0·3 ml. 0·1 *M* glucose as substrate, of suspension of *Bact. suboxydans* from growth on maize wort agar containing 2 % chalk. Temp. 30°. *p*H, 5·5.

Fig. 3. Oxygen uptake and CO_2 output, with 0·3 ml. 0·05 *M* calcium gluconate as substrate, of suspension of *Bact. suboxydans* from growth on maize wort agar containing 2 % chalk. Temp. 30°. *p*H, 5·5.

In most experiments the difference in volume between oxygen uptake and evolution of carbon dioxide increases beyond 330 μl. to about 450–470 μl. This additional excess of oxygen absorption may perhaps be accounted for by the partial oxidation of gluconic acid to 5-ketogluconic acid, $CH_2OH.CO(CHOH)_3COOH$, a reaction which has been observed for other acetic acid bacteria by several workers [Bernhauer & Schön, 1929; Hermann & Neuschul, 1931; Takahashi & Asai, 1931]. There are also the possibilities that 2-ketogluconic acid [Bernhauer & Görlich, 1935], 6-aldehydogluconic acid (*l*-glucuronic acid) [Takahashi & Asai, 1931; 1933] and saccharic acid may be formed. Another but less likely possibility is that there may be some oxidation process without evolution of carbon dioxide later on in the degradation of the glucose or gluconic acid molecule. However, the fact that 5-ketogluconic acid is a normal product of the glucose and gluconic acid metabolism of *Bact. suboxydans* in ordinary liquid culture points to this compound as being the most likely product in the experiments described above.

The view that gluconic acid is the first product of the action of the suspension of *Bact. suboxydans* grown on maize wort agar containing chalk is strengthened by the results obtained with 0·3 ml. of 0·05*M* calcium gluconate in place of the equivalent 0·3 ml. of 0·1*M* glucose as substrate in the Barcroft apparatus (Fig. 3).

Here the experimental results obtained are very similar to those obtained with glucose, with the significant exception that the difference between oxygen uptake and carbon dioxide output, that is, the absorption of oxygen unaccompanied by any evolution of carbon dioxide, rose only to about 120μl. after 5 hours. In the case of glucose, as stated above, the difference in most cases became stabilized at about 450–470μl., and this difference was usually attained in less than an hour. It will be noted that the two sets of figures for glucose and gluconic acid, i.e. 120μl. and 450–470μl., differ by 330–350μl., which is approximately equivalent to one atom of oxygen for 0·3 ml. substrate, that is, to the difference in oxygen content of the glucose and gluconic acid molecules.

Phosphatase activity of Bact. suboxydans.

Synthesis of phosphoric esters. All attempts to demonstrate the synthesis of hexosephosphates by the two types of suspension or their air-dried and acetone preparations gave negative results. The method employed was to make up flasks containing the following mixtures: 1 g. of air-dried or acetone bacteria or 1 ml. suspension, variable quantities of glucose ranging from 0·03 to 0·7 g., 0·07 ml. toluene and 2·5 ml. of 0·3*M* Na_2HPO_4 for experiments on the alkaline side of neutrality or an equivalent quantity of KH_2PO_4 for experiments in acid conditions. The total volume was brought up to 10 ml. in each case and the flasks incubated at 30°. The amount of inorganic phosphate was determined [King, 1932] after 5, 10, 20, 30, 60 min. and afterwards at wider intervals up to 48 hours. No definite decrease in organic phosphate was observed in any experiment, though there were slight deviations on either side of the original phosphate value. These negative results do not necessarily mean that no hexosephosphates are synthesized, as they may be broken down immediately they are formed.

Hydrolysis of sodium hexosediphosphate. Simon [1930, p. 277] demonstrated the ability of *Bact. suboxydans* to hydrolyse magnesium hexosediphosphate to the extent of over 80 % in 48 hours.

In order to compare the hydrolytic powers of the two types of suspension and their acetone and dried preparations, the cultures from which they were derived were grown under the same conditions and on the same batch of maize wort agar, with the exception of the addition of chalk to the chalk-containing medium.

Mixtures of the following composition were made up:

0·5 g. acetone or air-dried preparation or 2 ml. suspension, 5 ml. buffer solution, 1 ml. of $M/7{\cdot}5$ sodium hexosediphosphate solution, the whole being made up to 10 ml. Potassium hydrogen phthalate buffer solutions of *p*H 4·5 were employed, preliminary experiments having shown that the optimum *p*H for the liberation of inorganic phosphate was between 4 and 5. The $M/7{\cdot}5$ sodium hexosediphosphate solution was prepared by dissolving 1·16 g. of anhydrous sodium sulphate in 30 ml. water, grinding with 2·44 g. of barium hexosediphosphate (B.D.H.) and centrifuging. Solutions containing bacteria and buffer, buffer and hexosephosphate were made up as controls. The flasks were placed in a thermostat at 30° and gently agitated. Samples were removed approximately every 24 hours and estimated for inorganic phosphate by King's method [1932].

Table I gives the results obtained in one experiment in which the suspension and acetone preparation of the two types were compared. Table II gives other experimental results obtained after varying periods of incubation at 30°. In this table the results within each experiment are strictly comparable. The results are expressed as mg. of inorganic phosphorus in each ml. of the mixture. The maximum possible value, for complete hydrolysis, is 0·83 mg./ml.

Table I. *Hydrolysis of sodium hexosediphosphate by* Bact. suboxydans.

A represents suspension or acetone preparation from maize wort agar containing 2 % chalk.
B represents suspension or acetone preparation from maize wort agar without chalk.

Time hours	Suspension A dry weight 25 mg./ml.	Suspension B dry weight 23 mg./ml.	Acetone preparation A	Acetone preparation B
23	—	—	0·17	0·05
44	0·33	0·16	0·39	0·15
70	0·47	0·20	0·45	0·19
90	0·51	0·24	—	—
116	—	—	0·59	0·32

Table II. *Hydrolysis of sodium hexosediphosphate by* Bact. suboxydans.

	Culture grown on maize wort agar containing 2 % chalk		Culture grown on maize wort agar with no chalk	
Experiment	Air-dried preparation	Acetone preparation	Air-dried preparation	Acetone preparation
No. 1 after 1 day	0·29	0·16	0·06	Trace
No. 1 after 3 days	0·43	0·53	0·10	0·10
No. 2 after 3 days	—	0·53	—	0·28
No. 3 after 1 day	0·44	—	0·09	—
No. 4 after 2 days	0·40	—	Nil	—

An examination of Table II shows that the results obtained were variable, but it is clear that there is a very definite difference between the two types in their power of forming inorganic phosphate from sodium hexosediphosphate. In one experiment (No. 4) and in one only, the air-dried preparation of the culture grown without chalk liberated no free inorganic phosphate, but in the other experiments the preparation derived from this medium showed some phosphatase activity but considerably smaller than that of the preparation derived from the maize wort agar containing chalk. It is therefore evident that the strong oxidative ability possessed by suspensions of *Bact. suboxydans* from cultures grown on maize wort agar containing 2 % chalk is accompanied by considerably higher phosphatase activity than that possessed by the suspensions of restricted oxidative capacity derived from maize wort agar containing no chalk.

The effect of subculturing Bact. suboxydans *of either type on to the characteristic medium of the other.*

In order to determine whether the properties acquired by suspensions of *Bact. suboxydans* by growth on chalk maize wort agar were permanent or not, a culture which had been grown on the chalk medium for several generations was transferred to ordinary maize wort agar. Suspensions of the growth were made and examined for oxygen uptake and carbon dioxide output, while air-dried and acetone preparations of the suspensions were examined for phosphatase activity. All showed the characteristic properties of the type normally derived from maize wort agar. Similarly a culture which had been grown on ordinary maize wort agar for a long time immediately acquired the properties of the other variety when transferred to the chalk medium.

It is evident from this that the considerable modification induced in the enzyme system by the addition of chalk to the culture medium is a temporary variation and not a permanently acquired character.

SUMMARY.

1. *Bact. suboxydans*, an acetic acid organism characterized by its property of restricted oxidation in aerobic conditions, is found to possess a strongly oxidizing enzymic system when grown on maize wort agar containing 2 % chalk.

2. Washed suspensions of the growth from maize wort agar without chalk are dirty white in colour and oxidize glucose to gluconic acid but no further at the optimum *p*H 5·5. There is no evolution of carbon dioxide. These suspensions have no action on gluconic acid.

3. Washed suspensions from maize wort agar containing chalk are pink and give a much larger and more rapid oxygen uptake with glucose as substrate than the other type of suspension, as well as a considerable evolution of carbon dioxide. Gluconic acid is first produced but is immediately oxidized with evolution of carbon dioxide equal to the oxygen absorbed in this reaction. Some keto- or aldehydo-gluconic acid, probably 5-ketogluconic acid, may also be formed. The action of this type of suspension on gluconic acid is very similar to that on glucose, and the difference in oxygen uptakes unaccompanied by evolution of carbon dioxide is accounted for by the difference of one atom of oxygen between the glucose and gluconic acid molecules.

4. No synthesis of hexosephosphates by suspensions or air-dried and acetone preparations of either type could be detected.

5. The suspensions and air-dried and acetone preparations of the cultures grown on the chalk-containing medium were found to possess a much greater capacity for hydrolysing sodium hexosediphosphate than the corresponding suspensions and preparations from the maize wort agar without chalk.

6. The modification in enzymic properties produced by the chalk is a temporary variation and not a permanently acquired character.

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CCLXIII. THE UTILIZATION BY THE RAT OF VITAMIN A AND CAROTENE ADMINISTERED IN DIFFERENT MEDIA.

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(*Received 27 August 1936.*)

Before the adoption of the International Standard of reference for vitamin A, a large amount of work was carried out by several workers to determine the best solvent for its administration to experimental animals. A full account of this work has been given in the Medical Research Council's Special Report, No. 202 [1935]. The general conclusion was that the Standard (a particular sample of β-carotene) was more effective when administered in coconut oil or arachis oil (particular samples only) than when administered in olive oil, hardened cottonseed oil, ethyl laurate or liquid paraffin. There was some evidence from colorimetric determinations that the carotene was less stable in the latter solvents than in the samples of coconut oil used. Baumann & Steenbock [1933, 2] found by colorimetric determination less destruction of carotene in a refined cotton seed oil than in any other solvent. The possibility that carotene might be utilized differently in different solvents was mentioned in the Medical Research Council's Report.

It seems admissible to test this possibility by the following consideration. If the carotene in a particular solution is imperfectly utilized by the animal, the extent to which different animals utilize it would, presumably, vary. That is, if less than 100 % of the carotene were utilized, it could not be expected that all animals would utilize exactly the same proportion. On this view, therefore, the variation in response of animals given carotene in these "poor" solvents would be greater than that of animals given carotene in the "best" solvent if the "best" solvent owes its superiority to the better utilization of the carotene in it. A measure of this variation can be made by the generally accepted method of estimating the standard deviation (σ) of the test.

Since there are not enough figures available to make a reliable comparison between the variation in response of animals given carotene in coconut oil and that of animals given carotene in other oils, other comparisons of a similar nature have been made from the large amount of material available from the carrying out of tests in this laboratory. Comparisons which at once suggested themselves as useful for this purpose and for which there was sufficient material, were (*a*) cod liver oil and plant tissues; (*b*) butter and margarines containing a vitamin concentrate. The vitamin A of cod liver oil is regarded as true vitamin A whilst that of plant tissues is probably entirely in the form of carotene. The vitamin A of butter is probably largely true vitamin A (about 15 % of it was found to be carotene by Baumann & Steenbock [1933, 1]), whilst that of the margarines is also true vitamin A but is contained in different solvents, mostly vegetable oils. The examination of the standard deviations of the responses to these different sources of vitamin A has failed to show any real differences, and it therefore seems probable that the differences in results obtained by the solution of carotene in different oils cannot be due to differences in utilization of the carotene or vitamin A contained in them.

The general estimate of the standard deviation of a single determination (σ) *for the response of rats to a dose of vitamin A.*

A general estimate of the standard deviation of a single determination (σ) for the response of rats to a dose of vitamin A given daily for 5 weeks after they had ceased to grow on a diet deficient in that factor was published by Coward [1932] from results obtained during the period November 1929 to December 1931. The method of calculating σ was fully described in that paper. From these same experiments the standard deviation of the response of rats during the first three weeks of the test has been calculated (Table I). The tests have since been carried on with no avoidable differences, and from time to time the standard deviation of the three weeks' test has been determined from the whole of the results available. These are collected in Table I. No explanation of the rise in the value of σ in 1932 and 1933 is suggested, or of the subsequent fall in 1934, 1935 and 1936. The values are given to show that the variation in the response of animals to apparently the same treatment may itself vary from time to time. The average value for σ is given as a general estimate of its value for tests carried out in this laboratory.

Table I. *Variation in response to doses of vitamin A.*

(The whole of the tests from November 1929 to July 1936.)

Serial No. of tests	Dates (approx.)	No. of rats	No. of groups	Σd^2	σ^2	σ
		Bucks				
1–108*	Nov. 1929–Dec. 1931	677	112	70,579	126·69	11·26
109–189	Jan.–Dec. 1932	337	72	48,185	182·19	13·50
190–303	Jan. 1933–Sept. 1934	449	111	72,985	216·52	14·71
304–402	Oct. 1934–Oct. 1935	409	92	39,404	124·30	11·15
403–514	Nov. 1935–July 1936	377	95	28,931	102·58	10·13
1–514	Nov. 1929–July 1936	2249	482	260,084	147·19	12·13
		Does				
1–108*	Nov. 1929–Dec. 1931	719	112	43,990	72·47	8·51
109–189	Jan.–Dec. 1932	468	82	51,626	133·69	11·56
190–303	Jan. 1933–Sept. 1934	610	116	48,917	99·02	9·95
304–402	Oct. 1934–Oct. 1935	529	97	35,240	81·57	9·03
403–514	Nov. 1935–July 1936	486	108	31,864	84·30	9·18
1–514	Nov. 1929–July 1936	2812	515	211,637	92·14	9·60

* Including 4 inserted afterwards.

In addition a calculation has been made of the standard deviation of the response in all tests made on cod liver oil Z since it was adopted as a subsidiary standard of reference in this laboratory in November 1932. These results are included in the general estimate of the standard deviation, of which they form only about 16 %. The value of σ for cod liver oil is, according to both male and female rats, equal to the average for all the tests (Table II).

Table II. *Variation in response to vitamin A in cod liver oil Z.*

(November 1932 to July 1936.)

	No. of rats	No. of groups	Σd^2	σ^2	σ
Bucks	347	82	38,330	144·64	12·03
Does	452	85	32,498	88·55	9·41

Comparison between the variation in response to doses of vitamin A (a) *in cod liver oil and* (b) *in plant tissues.*

Since there is evidently some influence affecting the variation in response of the whole stock of animals from time to time, a comparison of the variation of two groups of animals can only be made safely with those animals which are on test simultaneously, so that whatever is affecting the variation must affect both groups equally. During the last twelve months we have been estimating the vitamin A contents of a large number of vegetable tissues (fruits, vegetables, pulses etc., some of them dried but most of them fresh) by comparison with our cod liver oil Z. These, together with a few more tests carried out previously, have given us sufficient material for this comparison. Our procedure for such comparisons is to prepare large numbers of rats by giving them the vitamin A-free diet, starting several litters two or three times a week, so that we have a continuous supply of rats becoming steady in weight on a shortage of vitamin A. As the rats become ready for test, they are divided into groups, one to be given a dose of the standard, another to be given a dose of the test substance. We do not regard it as essential that litter-mates should be used for comparisons, for we have determined (unpublished results) that the variation in response of litter-mates to the same dose of vitamin A, given when the rats have become steady in weight, is not less than that of rats from different litters. We are fully aware that the members of different litters have different reserves of vitamin A, but we consider that our procedure of just exhausting these reserves reduces the rats from different litters to a fairly similar condition. Naturally, in prophylactic tests or in partially prophylactic tests, litter-mate comparison is essential.

In assigning the rats which are ready for test to the different groups we think it is highly important that as far as possible equal numbers of rats should be assigned to the different groups each day. Whatever may be the various causes of the variation in response, those which may be felt from day to day (such as changes in temperature shown by Tourtellotte & Bacon [1935] to have an effect in vitamin D experiments) must be allowed to have equal influence on the different groups. If our supply of rats ready for test is good, we may compare two or even three test substances with the standard, putting the rats into the groups for (*a*) standard, (*b*) test A, (*c*) test B, and (*d*) test C respectively in regular sequence.

With the comparison controlled in this way, we have found that, according to the male rats of the tests, the variation in response to the vitamin A of cod liver oil is the same as to that of plant tissues, whilst according to the female rats, the variation in the response to the vitamin A of cod liver oil is a little larger than to that of the plant tissues but not significantly so (Table III).

Table III. *Comparison between cod liver oil and vegetables tested at the same time with regard to the variation in response to vitamin A.*

(May 1933 to July 1936.)

		No. of rats	No. of groups	Σd^2	σ^2	σ
Cod liver oil	Bucks	163	36	14,245	112·17	10·59
	Does	193	38	14,727	95·01	9·75
Vegetables	Bucks	227	60	18,793	111·20	10·54
	Does	294	72	16,531	74·46	8·63

Comparison between the variation in response to doses of vitamin A in (a) *butter, and* (b) *margarine containing a vitamin concentrate.*

For some years we have been making a comparison almost every month between a sample of a "vitamin margarine" and a sample of dairy butter, both bought at the same time and in the open market. Thus we have ample material from which to judge the variation in response to doses of vitamin A (*a*) as contained in butter and (*b*) as contained in this margarine. The comparisons were always made as described in the last section of this paper, i.e. they were made by simultaneous tests of the two products. The result may be seen in Table IV.

Table IV. *Comparison between butters and vitaminized margarines tested at the same time, with regard to variation in response to vitamin A.*

(January 1930 to July 1936.)

		No. of rats	No. of groups	Σd^2	σ^2	σ
Butters	Bucks	257	54	33,007	162·60	12·75
	Does	315	56	24,483	94·53	9·72
Margarines	Bucks	281	67	28,042	131·04	11·45
	Does	371	69	30,300	100·66	10·03

The standard deviation (σ) of a single determination according to the male rats was rather higher for the butters than for the margarines, but according to the female rats it was rather higher for the margarines than for the butters. Thus it must be concluded that there is no greater or less variation in the utilization of the vitamin A of butters than in that of the margarines containing the vitamin A concentrate.

SUMMARY.

Carotene has been found by several workers to be most effective as vitamin A on experimental animals when administered in coconut oil. This may be due to partial destruction in the "poorer" oils which has been demonstrated colorimetrically, but it has also been suggested that it may be partially due to a less complete utilization from the poorer oils.

If this is so, then it is to be expected that the utilization, being less than complete, would vary between different animals. This would bring about a greater variation in the response to a given dose of carotene than that which is observed normally when, presumably, utilization is complete. Comparisons of the variations in response to doses of carotene or vitamin A in different solvents or from different sources should, therefore, if carried out under strictly controlled conditions, indicate differences in utilization of the vitamin.

Comparisons of the variation in response to (*a*) the vitamin A in cod-liver oil and that contained, probably wholly as carotene, in plant tissues (for which 877 rats were used), and (*b*) the vitamin A (partly carotene) of butter and the vitamin A of margarines containing a vitamin concentrate (for which 1224 rats were used) show no significant differences.

It is therefore concluded that there is no difference in the powers of animals to utilize vitamin A or carotene either as supplied to them in cod liver oil or in

plant tissues, or as supplied to them in butters or as concentrates in various vegetable oils. The causes of the difference in effectiveness of carotene dissolved in different oils must be sought elsewhere.

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CCLXIV. A NOTE ON THE IDENTITY OF THE INDOPHENOL-REDUCING SUBSTANCES IN BRAIN TISSUE.

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Harris [1933] concluded from the results of biological tests that only about one-third of the material in Jensen rat sarcoma which reduces 2:6-dichlorophenolindophenol is ascorbic acid. Later Young & Mitolo [1934] and Mitolo [1934] brought forward evidence which they believed to indicate that brain tissue also contains an indophenol-reducing factor (I.F.) which is not ascorbic acid. Recent investigations have cast doubt on the conclusion that tumour tissue contains an I.F. other than ascorbic acid [Woodward *et al.* 1936; Watson, 1936; Kellie & Zilva, 1936]. The experiments described in this paper suggest further that the evidence previously believed to demonstrate the existence of a non-ascorbic acid I.F. in brain tissue is inconclusive.

The evidence previously presented that brain tissue contains such a substance [Young & Mitolo, 1934; Mitolo, 1934] was as follows:

Substance in crude brain tissue extracts	Ascorbic acid
Readily reduces acid ammonium molybdate at room temperature	Does not readily do so
Does not reduce ammoniacal silver nitrate at room temperature	Reduction at room temperature instantaneous
Insoluble in acetone	Freely soluble in absolute acetone
Precipitated by mercuric acetate	Not so precipitated
No antiscorbutic activity	Antiscorbutic

The validity of the non-biological evidence given above that the substance present in crude brain extracts is not ascorbic acid is undermined by the following observation. Prolonged aeration, in alkaline solution, of a crude brain extract results in the complete disappearance of the I.F. If the reaction is then adjusted to its previous value (about pH 6) and pure ascorbic acid added in amount calculated to be equal to the I.F. destroyed, the properties of the resulting solution are similar to those of a crude brain extract. In other words, if ascorbic acid is added to crude brain extracts, it assumes the chemical and physical properties of the unknown I.F. The question of the biological evidence, which is unsatisfactory, will not be considered in this paper. It may be stated, however, that the results have been inconsistent. The crude brain extracts used in the biological tests necessarily had a high solid content because of the low concentration of the I.F. in ox brain. They were therefore somewhat unpalatable to the guinea-pigs receiving a scorbutic diet. Some indication was obtained of a definitely deleterious effect of these extracts on the growth of the animal before symptoms of scurvy appeared. It is clear that, when such highly concentrated crude tissue extracts are used, interpretation of the results must be cautious.

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[1] Beit Memorial Fellow.

EXPERIMENTAL.

Ox brains obtained from the slaughter-house within a short time of the death of the animal were used in the majority of these experiments. In many cases confirmatory results were obtained with brain tissue from other species.

Ascorbic acid and the I.F. in brain were estimated by the reduction of 2:6-dichlorophenolindophenol, using the technique of Birch *et al.* [1933]. The indicator was standardized against pure ascorbic acid (Hoffmann La Roche and Co.) which was itself checked against standard iodine solution. The figures for I.F. are expressed as mg. equivalent of ascorbic acid.

Crude brain extract was prepared by rapidly mincing the brain tissue into 1·5 volumes of absolute alcohol and extracting for 24 hours at 0° with occasional stirring. The extract was then filtered off and the residue extracted twice with 70 % alcohol. The alcoholic extracts were combined and evaporated *in vacuo* at an outside temperature of 30–40° to such a volume that 1 ml. contained about 1 mg. equivalent of I.F. "Inactivated crude extract" was prepared by adjusting the *p*H of the crude extract to about 10 and bubbling air through briskly until no reduction of indophenol reagent could be detected. The reaction was then adjusted to its original value. "Reconstituted crude extract" was prepared by adding pure ascorbic acid to "inactivated crude extract" in amount equivalent to the I.F. removed by aeration.

Table I.

Reagent (5 ml. in all cases)	1 mg. equivalent crude extract	Equivalent inactivated extract	1 mg. pure ascorbic acid	"Reconstituted extract"
8 % ammonium molybdate	Rapid reduction	0	0?	Rapid reduction
8 % ammonium molybdate + 1 ml. 1 % KH_2PO_4	Rapid reduction	0	Rapid reduction	Rapid reduction
Ammoniacal silver nitrate	Very slow blackening	0?	Instantaneous reduction	Very slow blackening
Ammoniacal silver nitrate + 10 mg. glutathione	Very slow blackening	0?	Very slow blackening	Very slow blackening
Acid (acetic) silver nitrate	Fairly rapid reduction	0	Rapid reduction	Fairly rapid reduction

Reducing tests are summarized in Table I.

Acetone-solubility. To 10 ml. of crude extract, containing 15·6 mg. equivalent of I.F., 95 ml. of acetone were added. The precipitate was redissolved and the precipitation repeated twice. The final precipitate contained 11·9 mg. equivalent of I.F. The combined filtrates were freed from acetone by distillation *in vacuo* and found to contain 0·82 mg. equivalent of I.F.

A repetition of this experiment using "reconstituted crude extract" gave the following results: precipitate contains 11·4 mg.: filtrate contains 0·35 mg. ascorbic acid.

Mercuric acetate precipitation. To 10 ml. of neutral crude extract were added 18·75 ml. of 20 % mercuric acetate solution. Precipitate and filtrate were separately freed from mercury by H_2S and the H_2S removed by evacuation combined with a stream of nitrogen. The precipitate contained 2·4 mg. equivalent of I.F.; the filtrate contained 5·4 mg. equivalent of I.F.

A repetition of this experiment with "reconstituted crude extract" gave the following results: precipitate contained 3·0 mg.; filtrate contained 5·5 mg. ascorbic acid.

Biological tests. A comparison of the antiscorbutic activity of ascorbic acid or orange juice with that of the I.F. in crude brain extracts has been made by determining the ability of these substances to prevent the appearance of scurvy in guinea-pigs receiving a scorbutic diet. The symptoms of scurvy were assessed on the growth curve and on the general post-mortem appearance. In one experiment the daily administration of 6 mg. was ineffective in preventing the appearance of scorbutic symptoms, in another the feeding of 3 mg. equivalent was effective. In general six animals were used in each group, and as far as possible the conditions were similar in all cases, but over a considerable number of experiments the results were inconsistent.

Absorption spectrum. "Crude extract" at *p*H 7 gave an absorption band with a (somewhat broad) maximum at 255 mμ. This might have been considered additional evidence that the I.F. differed from ascorbic acid, which in neutral aqueous solution has a well-defined peak at 263 mμ. Fractionation of "crude extract" with lead acetate followed by removal of lead from the separated fractions by H_2S showed that 71 % of the recovered I.F. had been precipitated by basic lead acetate, and now exhibited a band at 263 mμ, whereas the filtrate from this precipitation contained only 14 % of the recovered I.F. but possessed a strong band with maximum absorption at 247 mμ. That the original band at 255 mμ was constituted largely, if not entirely, of two bands, one at 247 mμ and the other at 263 mμ received support from the observation that "inactivated crude extract" exhibited a strong band with maximum at 247 mμ.

DISCUSSION.

The simulation of the properties of the I.F. in crude brain extracts by added ascorbic acid definitely invalidates the chemical evidence on which the existence of a non-ascorbic acid I.F. was deduced. It is now known that the reducing properties of ascorbic acid are greatly modified by the presence of glutathione [Emmerie, 1934; de Caro & Giani, 1934] and other substances present in tissue extracts [Mawson, 1935].

The facts presented in this note cannot be taken as evidence that the I.F. in brain tissue is entirely ascorbic acid. Nevertheless the chemical evidence is such that there is no reason to believe that an indophenol-reducing factor other than ascorbic acid exists in brain tissue.

SUMMARY.

1. The properties of the indophenol-reducing substance in crude brain extracts differ in certain respects from those of pure ascorbic acid.

2. If pure ascorbic acid is added to an "inactivated" crude brain extract the properties of the resulting solution resemble those of the crude brain extract.

3. There is therefore no reason to believe that the indophenol-reducing substance in crude brain extracts is other than ascorbic acid.

I wish to express my thanks to Dr R. D. Heard, of the University of Toronto, who carried out a number of the biological tests and to Dr R. J. Macwalter, who determined the absorption spectra.

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CCLXV. REACTIONS OF PYRUVIC ACID WITH THIOLACETIC ACID AND CYSTEINE.

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(*Received 27 July 1936.*)

BAUMANN [1885, 2] observed that the action of ethyl-, phenyl- and *p*-bromophenyl-mercaptans on pyruvic acid led to the exothermic formation of substances which could be recrystallized from benzene and possessed well-defined melting-points. Though stable in the solid state and in benzene solution, these compounds were rapidly broken down to their original constituents when dissolved in water, and were regarded by Baumann as having the general structure $RS.C(OH)(CH_3).COOH$. When treated with dry HCl the thiophenylpyruvic acid passes into the mercaptol $(RS)_2C(CH_3).COOH$. The mercaptals and mercaptols [Baumann, 1885, 1, 2] have been extensively studied both by Baumann and his school and by other investigators, but the unstable "hemimercaptals" and "hemimercaptols" were little investigated until the recent work of Schönberg & Schütz [1927], Levi [1932] and Schubert [1935; 1936] appeared. Their work is of interest both from the point of view of the chemistry of the formation of mercaptals [Fromm, 1889; Levi, 1932] on which the present paper and recent work [Giršavičius & Heyfetz, 1935; 1936, 1] throw some light, and also from a biological aspect. Lohmann's [1932] well-known discovery that the transformation of methylglyoxal into lactic acid by glyoxalase requires the presence of reduced glutathione as a necessary and specific co-enzyme, together with the observations of Kühnau [1931], of Lohmann [1932] and of Jowett & Quastel [1933], that glutathione reacts in aqueous solution with methylglyoxal to form a fairly unstable compound, have led to the hypothesis [Jowett & Quastel, 1933] that a hemimercaptal-like compound of methylglyoxal with glutathione forms a necessary intermediate stage in the enzymic reaction. Further evidence for this view has been advanced by Platt & Schroeder [1934] and by Giršavičius & Heyfetz [1936, 2]. Possibly the biological significance of reactions of this type may extend beyond their participation in glyoxalase action [see Kühnau, 1931; Bersin, 1935].

In the following experiments the compounds formed between pyruvic acid on the one hand and thiolacetic acid [Baumann, 1885, 2; Bongartz, 1886] or cysteine on the other have been investigated more closely, the process of their formation and its reversal being studied.

Reversible combination of pyruvic with thiolacetic acid.

Thiolacetic acid (2·12 g.) and pyruvic acid (2·03 g.) in substance were mixed; the mixture became hot, then set to a mass of white crystals mixed with a good deal of viscous liquid. After cooling, the product was stirred up with ether and filtered through a sintered glass funnel. Yield: 2·63 g. (64 %) of crystalline substance. The ether washings (20 ml.) gave on evaporation a small additional crop. Ice cooling during the reaction did not appreciably alter the yield.

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[1] The work described in this paper was carried out in 1933, while both authors were working at the Cambridge Laboratory.

The following investigation of the crystalline compound shows that it is made up of the two reactants in equimolecular amounts and that it is exceedingly labile in aqueous solution.

Titration of —SH group. 61 mg. of the compound were dissolved in 5 ml. of ice-cold water and immediately titrated with 0·0996 *N* iodine to the first stable yellow tint. Iodine uptake was as rapid as speed of titration permitted. 3·50 ml. were required. Calculated for complete oxidation of the thiol group: 3·40 ml. [cf. Lucas & King, 1932].

Titration of —CO group. 59 mg. titrated according to Cook & Clift [1932]. Found: 6·58 ml. iodine. Calculated: 6·58 ml.

Isolation of components. (1) 5·647 g. of the addition product were dissolved in 30 ml. of water and 5 g. of $NaHSO_3$ added. The solution was repeatedly extracted with ether and the combined ethereal extracts dried over Na_2SO_4; evaporation of the ether left 2·95 g. of oily substance (102 % of original thiolacetic acid). Vacuum distillation (102–112°/10–16 mm.) gave 2·04 g. (70 %) of thiolacetic acid, identified iodimetrically and acidimetrically. (2) 0·4975 g. of the compound in 10 ml. of water was treated with 50 ml. of 2*N* HCl, containing 0·8 g. of 2 : 4-dinitrophenylhydrazine. The precipitate was twice purified by dissolving in *M* Na_2CO_3 and reprecipitating by acidification. Yield: 0·752 g., corresponding to 94·1 % of the theoretical amount of pyruvic acid dinitrophenylhydrazone. In another experiment 91·6 % was obtained.

Considering the losses involved in the isolation methods, these results, together with the above described titrations, demonstrate the easy dissociation in aqueous solution of the addition compound. No conclusion can however be drawn from these experiments as to whether the compound is actually incapable of existing in aqueous solution, or whether it attains an equilibrium, dissociating more or less according to the dilution and the extent to which one or other component is removed.

Reaction of pyruvic acid with cysteine.

Titration methods were incapable of telling us anything about the state of the pyruvic-thiolacetic compound in aqueous solution, or even whether any reaction takes place, when the components are mixed in solution. The lower reactivity of the products obtained by the interaction of pyruvic acid with cysteine permits a more profitable application of iodine titration. We attempted also to obtain a clearer picture by a parallel study of the changes in rotatory power undergone by natural *l*-cysteine in presence of pyruvic acid.

The readings were taken with Hg green ($\lambda = 5461$ Å.) in a 2 dm. tube. The times were measured with a stopwatch and alternate readings were taken approaching from the right and from the left.

Fig. 1 shows the course of change of rotation of 0·2 *M* cysteine in aqueous solution in presence of 0·2, 0·4 and 0·6 *M* pyruvic acid. The solutions were always kept long enough for the rotation to reach a final value (several days). These end-values were: for 0·2 *M* pyruvic acid −5·32°, for 0·4 *M* −7·2°, and for 0·6 *M* −7·44°. The experiments were carried out at 26–27°.

Fig. 2 shows a number of curves obtained with cysteine and pyruvic acid in alcoholic solution (in view of the possible dissociating effect of water), at 37° ± 0·1° in a jacketed polarimeter tube. The cysteine concentration was again 0·2 *M* and those of pyruvic acid 0·2 and 0·4 *M*. The end-values, after several days in a thermostat at 37°, were: 0·2 *M* pyruvic acid −7·52°, 0·4 *M* −9·56°. An inspection of both sets of curves shows that they have their origin at some point corresponding to a weak negative rotation, which, as far as can be judged, is the more marked, the higher the pyruvic acid concentration. Since cysteine itself is weakly dextrorotatory (arrow in Fig. 1 shows the rotation of 0·2 *M* cysteine alone), we are clearly dealing with two successive effects: a practically instantaneous shift of rotation (of about 1° with our concentrations) with reversal of sign, succeeded by the gradual development of a strong laevorotation.

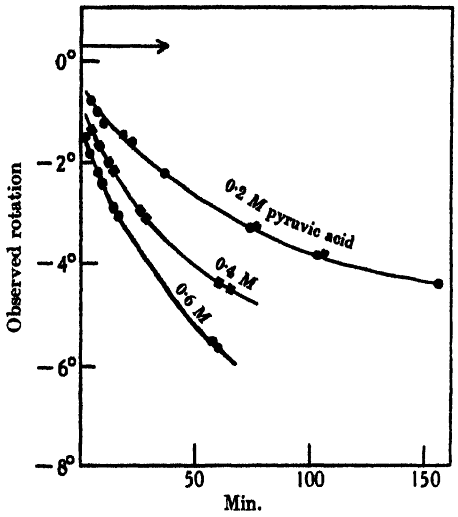

Fig. 1.

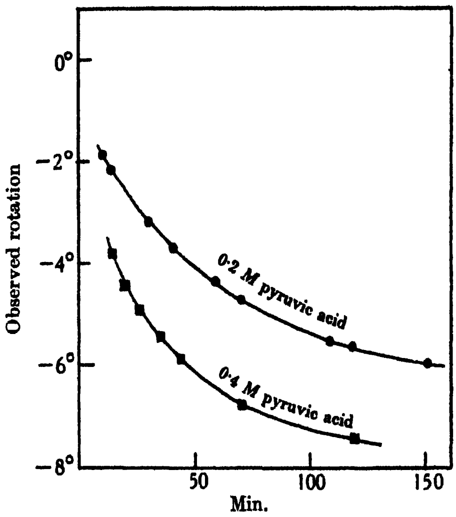

Fig. 2.

In view of the complexity of these reactions, of the initial shift of rotation which seems to imply a rapid first stage[1] and the variable end-point which seems to indicate the attainment of an equilibrium, it is not surprising that the reaction courses observed fail to fit the ordinary equations. With equimolecular concentrations of pyruvic acid and cysteine the bimolecular reaction constants increase with time; the unimolecular constant is reasonably uniform in the experiment in water, but rapidly falls in the alcoholic solution. In all the other experiments the bimolecular constant falls with time, the unimolecular constant of course even more so.

Some colour reactions.

An attempt to obtain some of the typical —SH reactions in presence of pyruvic acid provided additional evidence of a combination involving the sulphydryl group of the cysteine.

$FeCl_3$. Added to an alkaline solution of cysteine, $FeCl_3$ gives a purple colour, which fades on standing, but reappears on admitting oxygen [Harris, 1922; Michaelis & Barron, 1929]. To an alkaline (ammonia) mixture of 1 ml. *M*/4 cysteine + 2 ml. *M*/4 Na pyruvate was added 0·1 ml. *M*/100 $FeCl_3$. The purple colour faded more rapidly than in absence of pyruvic acid and reappeared less strongly on shaking; after repeating several times the cycle of reduction and reoxidation the colour fails to reappear again. In absence of pyruvic acid the process can be repeated almost indefinitely. If the cysteine and pyruvic acid are allowed to stand a short while before adding $FeCl_3$ the colour reaction is faint and rapidly vanishes irreversibly.

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[1] Unpublished observations by one of us with P. A. Heyfetz have demonstrated a similar phenomenon in the reaction of GSH with methylglyoxal (iodimetric titration).

Nitroprusside. In ammoniacal solution cysteine gives immediately the well-known purple colour; this slowly fades to a brownish yellow. Pyruvic acid, under the same conditions, gives a slowly developing blue colour. On adding nitroprusside to an ammoniacal solution of cysteine and pyruvic acid (in excess) a transient purple colour is observed, followed by the development of the blue pyruvic acid colour. Between the two a colourless interval is sometimes noticed, showing that the colour due to cysteine has actually vanished and is not merely covered by the deeper colour due to excess pyruvic acid.

Methylene blue reduction. The reduction of methylene blue by cysteine in alkaline solution is inhibited by pyruvic acid, though not very strongly (Thunberg tube).

Titration experiments.

Absolute alcoholic solutions of cysteine hydrochloride (0·19 *M*) and of pyruvic acid ($2\frac{1}{2} \times 0·19 = 0·475$ *M*), or mixtures of the two solutions were added by means of 0·5 or 1 ml. Ostwald pipettes to ice-cold 1–2 *N* HCl containing 1 ml. of 0·1014 *N* iodine and one drop of starch solution. The excess iodine was titrated with 0·1001 *N* $Na_2S_2O_3$ from a microburette allowing an accuracy of 0·001 ml.

(1) 3 ml. of 1·3 *N* HCl + 0·5 ml. cysteine solution + 0·2 ml. pyruvic acid solution were mixed ice-cold. The mixture was kept in ice for various periods before adding iodine and (as rapidly as possible) titrating. As in all further experiments, the iodine consumption is given in terms of 0·1 *N* iodine. Cysteine alone (no pyruvic acid): 0·958, 0·955 ml.; with pyruvic acid: after 2 min. 0·907 ml., after 10 min. 0·881 ml., after 15 min. 0·789 ml.

(2) 15 ml. of the cysteine solution + 6 ml. of the pyruvic acid solution (that is, an absolute alcoholic solution, 0·136 *M* in each of these substances) were kept for 4 days. 0·5 ml. samples were added to a mixture of 1 ml. 2 *N* HCl + 1 ml. 0·1 *N* iodine, cooled in a freezing mixture to −5° to −6°; on adding the alcoholic solution the temperature rose to about 0°. The titration vessel containing the mixture (a short wide tube with a tapered bottom) was transferred to a beaker containing ice in dilute brine, so that its temperature up to and during the titration was kept at −2° to 0°. At the moment of adding the cysteine-pyruvic acid mixture to the iodine a stopwatch was started, which was stopped at the

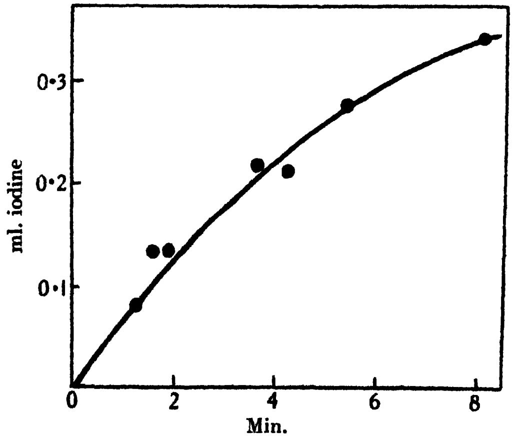

Fig. 3.

end of the titration. The titration was started after the iodine had been acting for various periods. Fig. 3 shows the course of iodine uptake up to 8 min. The contrast with the experiments with thiolacetic acid, where the whole of the iodine corresponding to the —SH introduced is immediately consumed, is striking.

It is not clear from these results whether the gradual uptake of iodine is entirely due to its action on the compound, or whether a certain amount of dissociation takes place spontaneously when the alcoholic mixture is added to water + HCl. The following experiment was designed to answer this question. 0·5 ml. of the alcoholic mixture of pyruvic acid and cysteine was added to 1 ml. HCl + 1 ml. iodine, as above, or else only to HCl, the iodine being added some time later. The total times up to the end of the titration, were kept as nearly as possible alike.

	Iodine uptake ml.
Iodine at once. Titration finished in 2 min. 12 sec.	0·082
Iodine at once. Titration finished in 8 min. 14 sec.	0·213
Iodine added after 6½ min. Total time 8 min. 15 sec.	0·097

In the following experiment the alcoholic solution was added to 4 ml. 2 N HCl + 3 ml. of water; iodine present or added later.

	Iodine uptake ml.
Iodine at once. Titration finished after 7 min.	0·476
Iodine after 6½ min. Total time 7 min. 4 sec.	0·173

Little, if any, sulphydryl is free in the aqueous HCl solution in absence of iodine. This accords well with the polarimetric observations, which showed that the reaction follows much the same course in water and in alcohol. (It must be noted, that on adding the alcohol mixture to the dilute HCl not only does a change in the nature of the medium take place, but also a considerable change in volume). Judging from the second series of figures higher dilution increases the effect of the iodine and perhaps has itself a certain dissociating effect.

CONCLUSIONS.

Giršavičius & Heyfetz [1935] have recently studied in some detail the reaction between glutathione and methylglyoxal in aqueous solution. Two main conclusions were reached: (*a*) a true equilibrium is established between the free components and the reaction product; (*b*) the reaction rates in both directions depend on the *p*H, being slow in strongly acid solution and extremely rapid as the solution approaches neutrality (compare Giršavičius & Heyfetz [1936, 1] where this observation is elaborated). The results described in the present paper fit in with the view that here too reactions of the same kind as that between glutathione and methylglyoxal may take place, and that such differences as are found, for instance between cysteine and thiolacetic acid, are expressions of different reaction rates and equilibrium constants. Baumann [1885, 1, 2], in investigating the products of spontaneous reaction between pyruvic acid and various mercaptans, already mentioned the differences in their properties, including their stability. Great differences in the reactivity (reaction rates) of the —SH group, in accordance with the structure of the molecule of which it forms a part, are also mentioned by Michaelis & Schubert [1934]. It must be admitted, however, that the great difference in behaviour shown by the compounds of pyruvic acid with thiolacetic acid on the one hand and with cysteine on the other suggests the possibility of a different type of bond. Schubert [1935] has shown that with cysteine and with thiocarbamide[1]) methylglyoxal forms not addition, but cyclic condensation compounds, apparently involving the nitrogen as well as the sulphur. Recently [1936] he has extended this observation to the compounds of cysteine with certain aldehydes. It is true that Schubert claims [1936] that the compound of cysteine with pyruvic acid is an addition, not a condensation compound; but the reaction he describes proceeded only for one day at about 0° and may, perhaps, not have been complete. At any rate we consider that the nature of the compound finally formed between pyruvic acid and cysteine, a compound fairly stable to iodine and endowed with strong laevorotation, still remains to be settled.

SUMMARY.

Reactions taking place in the systems pyruvic acid + thiolacetic acid and pyruvic acid + cysteine were investigated. Pyruvic acid in substance combines exothermically with pure thiolacetic acid to form a white crystalline compound. Dissolved in water this behaves like a mixture of free pyruvic acid and free thiolacetic acid, both in its reactions with iodine or bisulphite and in the possibility of isolating the original components from the aqueous solution. Cysteine in aqueous or alcoholic solution reacts slowly with pyruvic acid, as shown polarimetrically and by iodimetric titration. The compound is reasonably stable, as judged from its behaviour towards iodine and some colour reagents. A hypothesis is outlined which connects these reactions with the recently investigated reaction of glutathione with methylglyoxal, and some alternative explanations of the reaction of pyruvic acid with cysteine are discussed.

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[1] The condensation compound of methylglyoxal with thiocarbamide seems to have been first obtained by Sjollema & Kam [1916].

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CCLXVI. ENZYMIC RACEMIZATION OF OPTICALLY ACTIVE LACTIC ACID.[1]

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(*Received 19 August 1936.*)

It has been shown in a previous publication [1932] that certain lactic acid bacteria which by themselves form optically active lactic acid produce inactive (*dl*) lactic acid when grown in association with the acetone butyl alcohol organism *Cl. acetobutylicum*. This was thought to be due to an associative modification of the metabolism of the lactic acid bacteria, causing them to produce both forms of lactic acid rather than the one form produced normally. It was stated that the formation of inactive lactic acid took place in association with *Cl. acetobutylicum* but not with the closely related organism *Cl. butylicum*.

Further investigation has shown that *Cl. butylicum* when grown together with lactic acid bacteria does lead to the production of inactive lactic acid. It has further been found that the racemization is caused by the butyric organisms themselves, rather than by their effect on the lactic acid bacteria. Finally, it has been shown that the racemization of optically active lactic acid by *Cl. acetobutylicum* and *Cl. butylicum* is enzymic in character.

EXPERIMENTAL.

Cultures and media. Pure cultures of the following organisms were used: *Strep. lactis*, R.; *Lactobacillus delbrückii*, 3; *Cl. acetobutylicum*, S.; and *Cl. butylicum*, 21 (*Cl. sp.* 21 in previous report). A 3 % glucose, 3 % malt sprouts medium containing an excess of calcium carbonate was used for associated growth of the lactic cultures and the butyric organisms and for some of the enzyme work, but a medium containing 0·5 % glucose and 0·5 % "tryptone" was also used in the latter experiments.

Analytical methods. Volatile and non-volatile acids were determined by steam-distillation and extraction with ether respectively, as described in a previous publication [Pederson *et al.* 1926]. The form of lactic acid recovered was determined as in the previous report and was based on the percentage of water of crystallization and the specific rotation of the zinc salts.

In every case the zinc salts were crystallized from 50 % alcohol to ensure as nearly complete recovery as possible. Over 90 % of the lactic acid in each sample was recovered as the salt. In this way truly representative samples for determination of water of crystallization and specific rotation were assured.

Effect of association of lactic acid bacteria and Cl. butylicum *on the form of acid produced.*

It was reported in the previous paper that *Cl. butylicum* had no effect on the form of acid produced in association with lactic cultures. This work has been

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[1] This work was supported in part by a grant from the Special Research Fund of the Graduate School.

repeated. The results in Table I show that the associated growth of *Cl. butylicum* with either *Strep. lactis*, R. or *L. delbrückii*, 3, caused the production of inactive lactic acid. The acid formed by the lactic acid bacteria alone was dextrorotatory. The previous negative results were probably due to the failure of *Cl. butylicum* to grow appreciably, as indicated by the low volatile acid production. Because of the sensitivity of this organism to acid it is difficult to establish a balance between the two cultures in a fermentation. These results indicate that *Cl. butylicum* has the same racemizing effect in associated growth on the form of lactic acid as does *Cl. acetobutylicum*. This would be expected from their close relationship.

Table I. *Effect of association with* Clostridium butylicum *on the form of lactic acid produced by bacteria.*

Organism	Water of crystallization of zinc salt of lactic acid %	Form of lactic acid produced
Strep. lactis	11·3	Dextro
Strep. lactis and *Cl. butylicum*	17·2	Inactive
Strep. lactis and *Cl. butylicum*	18·2	Inactive
L. delbrückii	12·9	Dextro
L. delbrückii and *Cl. butylicum*	18·1	Inactive
L. delbrückii and *Cl. butylicum*	18·2	Inactive
L. delbrückii and *Cl. butylicum*	18·19	Inactive

Racemization of d-*lactic acid by fermentation.*

In the previous paper [1932] it was suggested that the associated organism caused the lactic culture to produce both forms of lactic acid rather than only one as it did normally. An alternative explanation which was not considered at that time is that the lactic acid as formed is active but that the associated organism, *Cl. acetobutylicum*, brings about the racemization.

The validity of the latter hypothesis was tested by growing the butyric acid bacteria in a medium containing glucose (3 %), malt sprouts (3 %), calcium carbonate and the calcium salt of *d*-lactic acid. After several days' incubation the fermentations were stopped and the unfermented lactic acid was recovered and the form determined. Table II gives the results. In 2 days *Cl. acetobutylicum* destroyed only 12 % of the lactic acid and had only a slight racemizing effect. After 3 days, however, more volatile acid was produced, and 54 % of the lactic acid was recovered. It was entirely inactive. *Cl. butylicum* had

Table II. *Racemization of* d-*lactic acid* by fermentation.*

300 ml. medium: 3 % glucose, 3 % malt-sprouts, $CaCO_3$.

Organism	Period of fermentation days	Volatile acid as acetic g.	Lactic acid recovered g.	Lactic acid recovered %	Water of crystallization of zinc salt of recovered lactic acid %	Form of lactic acid recovered
None	Sterile	--	5·16	100	12·9	Dextro†
Cl. acetobutylicum	2	1·97	4·54	88	14·7	Mainly dextro
Cl. acetobutylicum	3	2·98	2·78	54	18·6	Inactive
Cl. butylicum	2	1·17	2·68	52	17·0	Mainly inactive
Cl. butylicum	3	1·53	2·25	44	18·1	Inactive

* Lactic acid added as calcium salt. † Specific rotation = −7·9°.

a similar effect. In 2 days 52 % of the lactic acid was recovered. It was mainly inactive. Only 44 % was recovered after 3 days and it was completely racemized. These results indicated definitely that the formation of inactive lactic acid in association was due to the direct action of the associated cultures on the active lactic acid produced by the lactic acid bacteria.

Enzymic racemization of lactic acid.

It seemed possible that the racemization of lactic acid by the butyric acid bacteria might be due to enzymic activity. Therefore, instead of fermenting calcium lactate directly, the cultures were grown for 24 hours and active lactic acid was then added as a solution of the calcium salts. Toluene and chloroform were added at the same time. The mixtures were incubated for 3 days at 37°.

Table III. *Enzymic racemization of active lactic acid.*

		Lactic acid				Recovered zinc lactate	
		Added		Recovered			
Medium* ml.	Treatment	Form	g.	g.	%	Water of crystallization %	Form of acid
Culture, *Cl. acetobutylicum*:							
300 (M)	Antiseptics	Dextro	5·40	5·72	105·0	18·2	Inactive
Culture, *Cl. butylicum*:							
300 (M)	Antiseptics	Dextro	5·16	4·54	88·0	18·2	Inactive
300 (M)	Antiseptics	Dextro	5·16	4·86	94·2	17·3	Inactive
75 (T)	Antiseptics	Laevo	0·82	0·74	90·5	18·1	Inactive
75 (T)	Antiseptics	Laevo	0·82	0·68	82·4	17·8	Inactive
300 (M)	Heated, 68° antiseptics	Dextro	5·40	5·18	93·0	13·1	Dextro
300 (M)	Heated, 87° antiseptics	Dextro	5·40	4·80	89·0	12·9	Dextro†

* (M) = malt-sprouts, 3 %; glucose, 3 %. (T) = "tryptone", 0·5 %; glucose, 0·5 %.
† Specific rotation of zinc salt, − 6·8°.

The results in Table III show that from 82 to 100 % of the added lactic acid was recovered and that in every case it had been completely racemized. Both cultures were effective and both *d*- and *l*-lactic acids were changed to the inactive form. Cultures of *Cl. butylicum* failed to bring about the change after they had been heated to 68 or 87°.

Racemization by components of racemizing enzyme system.

In order to determine something about the nature of the enzymes responsible for racemization of active lactic acid, *Cl. butylicum* was grown in 0·5 % glucose, 0·5 % "tryptone" medium. After 24 hours' incubation the cells were removed by centrifuging. This treatment threw out most of the cells. The cell-free medium and the cells were separately made up to the original volume of the culture with distilled water. Active lactic acid (calcium salt), toluene and chloroform were added to samples of the cell suspension, the cell-free medium and a mixture of the two. Sufficient distilled water was added to the samples containing only the cell suspension or the cell-free medium to make the final concentration of each component one-half that in the original culture before centrifuging. After 3 days' incubation at 37° the lactic acid was recovered and the form determined. The results are given in Table IV. Neither the cell suspension nor the cell-free medium alone had any effect on the form of lactic acid. A combination of the two, however, was effective, since the recovered lactic acid was inactive. Both *d*- and

Table IV. *Enzymic racemization of lactic acid by cells and medium of* Cl. butylicum.

	Lactic acid				Recovered zinc salt		
	Added		Recovered		Water of crystallization	Specific rotation	
Agent	Form	g.	g.	%	%	$[\alpha]^{25°}_{D}$ °C.	Form of acid
Cells	Dextro	2·7	2·4	89	13·3	—	Dextro
Cells	Dextro	2·7	2·3	85	13·2	−6·8	Dextro
Medium	Dextro	2·7	2·7	100	13·2	−7·0	Dextro
Medium	Dextro	2·7	2·5	92	13·0	−6·5	Dextro
Cells and medium	Dextro	2·7	2·6	97	17·0	—	Inactive
Cells and medium	Dextro	2·7	2·7	100	18·1	—	Inactive
Cells	Laevo	0·82	0·70	85	13·1	+7·8	Laevo
Medium	Laevo	0·82	0·69	84	13·4	+7·0	Laevo
Cells and medium	Laevo	0·82	0·71	86	17·8	—	Inactive

l-lactic acids were racemized by the combined cells and medium. This indicates that at least two factors are concerned in the racemization. One seems to be extracellular and the other intracellular.

Heat-stability of components of racemizing enzyme system.

The separation into two components of the enzyme system responsible for racemization of active lactic acid suggested that the system might be similar to an enzyme-coenzyme system. Since many coenzymes are heat-stable, the heat-stability of the component cell and cell-free medium fractions was investigated. The fractions were prepared as detailed before, and portions of each preparation were heated for 10 min. at 87°. Combinations of heated medium with unheated cells, and of heated cells with unheated medium were tested for their racemizing actions on *l*-lactic acid (Table V). The activity of the cell-free medium fraction was destroyed by the heat treatment. However, the active component in the cell suspension fraction seemed to be heat-stable. The zinc salt from one sample of recovered lactic acid had a water of crystallization content of 16·6 % indicating a mixture of active and inactive acids. The sample was fractionally recrystallized. The first crop of salt was the pure inactive form, and the last

Table V. *Heat-stability of components of racemizing enzyme system of* Cl. butylicum.

	Lactic acid			Recovered zinc lactate	
	Laevo-acid added	Recovered		Water of crystallization	
Components and heat treatments	g.	g.	%	%	Form of acid
Heated medium and unheated cells	0·82	0·80	98	13·1	Laevo*
Heated medium and unheated cells	0·82	0·72	88	13·4	Laevo
Unheated medium and heated cells	0·82	0·80	98	16·6†	Mixture, laevo and inactive
Unheated medium and heated cells	0·82	0·74	90	18·0	Inactive

* Specific rotation of zinc salt, +7·3°.

† Sample fractionally recrystallized: first crop: H_2O, 18·2 %; last crop: H_2O, 13·4 %; specific rotation, +7·8°.

crop was pure active salt. Another sample of recovered lactic acid had been completely racemized. By analogy with other enzyme systems, this racemizing enzyme system seems to be composed of an extracellular heat-labile enzyme and an intracellular heat-stable co-enzyme.

DISCUSSION.

The racemization of active lactic acid by enzyme systems of *Cl. acetobutylicum* and *Cl. butylicum* seems to explain fully the results obtained in the previous paper. During that work an attempt was made to separate the two associated organisms by a viscose membrane. No change in the form of acid was observed. A racemizing enzyme produced by *Cl. acetobutylicum* should have been incapable of penetrating such a membrane, and any lactic acid which diffused through the membrane into contact with the enzyme would probably have been completely fermented. The production of active lactic acid in a sterilized *Cl. acetobutylicum* culture on inoculation with a lactic culture was due to the destruction of the racemizing enzyme by heat.

The work of Hammer [1920] with cheese starters suggests that other organisms may cause the production of inactive acid in association with *Strep. lactis*. It seems possible that the general production of inactive lactic acid in starters and in commercial lactic acid fermentations may be due primarily to the racemizing activities of contaminating organisms, which may or may not themselves produce lactic acid. If such were the case, the racemization might be a function of the enzyme concentration and activity rather than of the numbers of contaminating organisms. The growth of the contaminants and the elaboration of the enzymes would not necessarily lower the yield of lactic acid materially.

Pasteur in his classical work on optical activity found that micro-organisms such as moulds were capable of producing active lactic acid from the inactive form by selective fermentation of one enantiomorph. This does not involve any actual change in the configuration of the asymmetric molecules. In contrast, the results in this paper show that some bacteria can change optically active lactic acid quantitatively into the inactive form. This racemization necessitates a reversal of the configuration of the asymmetric molecules. This enzymic Walden inversion proceeds until the two enantiomorphs are in equilibrium. The resulting lactic acid consists of a mixture of both forms present in equal quantities. The enzymic attainment of such a state of equilibrium between the two optical isomerides would explain the complete racemization of either the dextro- or the laevo-rotatory form of lactic acid.

SUMMARY.

Inactive lactic acid was formed in associated growth of *Strep. lactis* or *L. delbrückii* with the butyric acid organisms *Cl. acetobutylicum* or *Cl. butylicum*.

The production of inactive acid in associated growth is probably due to the racemizing effect of the butyric organisms on the lactic acid after it has been formed by the lactic acid bacteria. In the absence of the lactic acid bacteria both *Cl. acetobutylicum* and *Cl. butylicum* partially fermented active lactic acid and changed the remainder to inactive acid.

The racemization seems to be brought about by an enzyme system elaborated by *Cl. acetobutylicum* and *Cl. butylicum* since cultures of these organisms changed either *d*- or *l*-lactic acid quantitatively into *dl*-acid in the presence of antiseptics.

There seem to be at least two components of this racemizing enzyme system, one extracellular and the other intracellular. Neither cell suspensions nor cell-free media of *Cl. butylicum* were able to racemize active lactic acid, but the two components were effective in combination.

The extracellular component was found to be heat-labile (87°, 10 min.) and the intracellular fraction was stable to this heat treatment.

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CCLXVII. GROWTH FACTORS FOR BACTERIA.[1]

V. VITAMIN B_1, A GROWTH STIMULANT FOR PROPIONIC ACID BACTERIA.

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(*Received 19 August 1936.*)

IN a previous paper [Wood *et al.* 1936] it was shown that for growth and fermentation the propionic acid bacteria require an ether-extractable acid found in aqueous extracts of yeast, potatoes and liver and in corn steep water. This stimulant was found to be essential for growth on a synthetic medium containing ammonium sulphate as the source of nitrogen. Although fairly good growth was obtained with this combination, continued transfer gave inconsistent results. The organisms generally failed to grow after the 3rd or 4th transfer. However, the addition of amino-acids in the form of hydrolysed caseinogen greatly increased cell growth and fermentation and made repeated transfer possible with consistent results.

In the present investigation a second growth factor for the propionic acid bacteria has been found which stimulates growth particularly in the presence of amino-acids. This paper deals with the sources, preparation and properties of this accessory factor, which has been found to be identical with vitamin B_1.

EXPERIMENTAL.

Cultures. Pure cultures of six strains of propionic acid bacteria were used in this study. These were selected from those studied by Hitchner [1934] and on the basis of his classification are representative cultures. The following were used: *Propionibacterium pentosaceum* 11; *P. freudenreichii* 33; *P. jensenii* 54; *P. zeae* 56; *P. pentosaceum* 57; and *P. arabinosum* 61. All cultures were carried on Difco yeast extract medium and were transferred at least twice on ammonium sulphate medium before inoculating in order to deplete the inoculum of any growth factors contained in the original medium. *P. pentosaceum* 11 was used as a test organism for most of the investigations, as in previous work, but the findings were checked against the other cultures.

Medium and analytical methods. The supplemented synthetic medium developed in the previous work [Wood *et al.* 1936] was used throughout this investigation. This has the following composition: glucose 1 %; sodium acetate, 0·6 %; ammonium sulphate, 0·3 %; inorganic salts; ether extract of 3 g. yeast extract per 100 ml. Fermentation was followed by direct titration of the acid produced.

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[1] This work was supported in part by a grant from the Special Research Fund of the Graduate School.

[2] National Research Fellow in the Biological Sciences.

Effect of protein hydrolysates on fermentation. It has been shown in the previous paper that the basal medium with the addition of hydrolysed caseinogen gave a fermentation practically equivalent to crude yeast extract medium. As this was thought to be due to the amino-acids added, it seemed that two factors were necessary for good growth; viz. the ether-soluble acidic factor and some necessary or beneficial amino-acids. However, when the amount of hydrolysed caseinogen which was necessary for maximum fermentation was compared with the amount of crude yeast extract required, it was apparent that the yeast was much more potent. Furthermore, the optimum concentration of hydrolysed caseinogen was greatly in excess of the probable amino-acid requirements, judged by the amounts needed by other bacteria [Fildes & Richardson, 1935; Mueller, 1935]. Two explanations of this seemed possible; yeast extract may contain some particularly beneficial amino-acids which are present in caseinogen in only small amounts, or yeast extract may contain an active ether-insoluble factor other than amino-acids which is present in small amounts in caseinogen hydrolysate.

If amino-acids were concerned, it seemed probable that hydrolysates of proteins other than caseinogen might have very different effects owing to their differences in amino-acid content. Therefore sulphuric acid hydrolysates of gelatin, egg albumin and a sample of purified caseinogen (reprecipitated and washed until vitamin-free) were prepared according to standard procedures. These were added to the basal medium and their activities compared with that of the crude caseinogen hydrolysate. A sample of crude yeast extract was also hydrolysed in the same way and tested. The results (Fig. 1) show that one of the hydrolysed egg albumins (sample 1) and hydrolysed yeast extract were much more effective than the crude caseinogen hydrolysate. Gelatin was almost as effective as the caseinogen. However, purified caseinogen was much inferior to the crude caseinogen, and a second sample of egg albumin (Merck) was still less

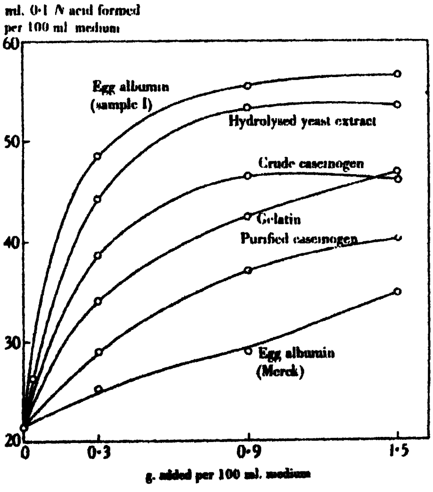

Fig. 1.

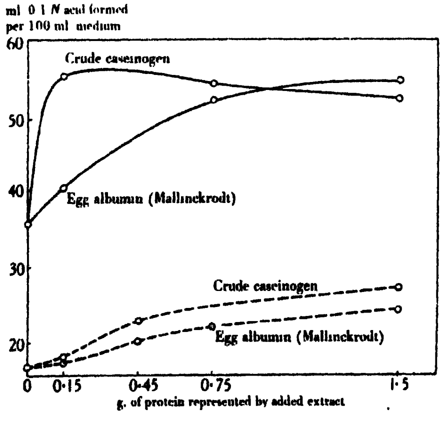

Fig. 2.

Fig. 1. Effect of hydrolysed materials on acid production.

Fig. 2. Stimulating effect of alcohol extracts of crude caseinogen and egg albumin on acid production.

—— 0·45 % hydrolysed purified caseinogen + 0·3 % $(NH_4)_2SO_4$. - - - 0·3 % $(NH_4)_2SO_4$.

effective. These results indicated that some factor other than amino-acids was primarily responsible for the superiority of yeast extract. In the first place there was no correlation between the known amino-acid contents of the different proteins and their observed activities; and in the second place there should have been no variation in the amino-acid composition of the two samples of caseinogen and the two egg albumin samples. It seemed probable that some factor in the hydrolysed proteins which was not a constituent of the protein molecule was primarily responsible for their activity. It has been found that 95 % alcohol and 85 % acetone extracts of crude yeast extract contain all the factors necessary for vigorous growth and fermentation. Therefore extracts of unhydrolysed crude caseinogen and egg albumin (impalpably powdered, Mallinckrodt) were made by continuous extraction with 95 % alcohol. The solvents were removed and aqueous solutions of these extracts were added in varying concentrations to the basal synthetic medium with and without hydrolysed purified caseinogen. The results (Fig. 2) show that these extracts contained some factor which was only slightly stimulating in the basal ammonium sulphate medium, but which had a very decided effect in the presence of hydrolysed purified caseinogen.

These results indicated quite conclusively that the stimulating factor was neither an amino-acid nor a constituent of the protein molecule, although its activity seemed to be associated with the utilization of amino-acids. It seemed possible that the original materials from which the proteins were derived might be better sources of the factor than the crude proteins. Therefore milk powder and dried egg white were extracted with alcohol and acetone. The extractions were carried out by covering the materials with the solvent, allowing to remain for 1 or 2 days with frequent shaking, and finally filtering off the insoluble residues. The solvents were removed and aqueous solutions of the extracted material were tested by adding them to the basal medium in the presence of hydrolysed purified caseinogen. It was found that the extracts of egg white were fairly active, whilst the milk powder extracts were even more active than the continuous alcohol extract of caseinogen. These extracts were just as potent in a basal medium containing 0·15% of hydrolysed purified caseinogen as in one containing 0·45%. The data in Fig. 3 show the effects of the three most active extracts on the production of acid in the presence of 0·15% hydrolysed purified caseinogen. The maximum stimulation was reached with a very low concentration of these extracts. The units chosen were those amounts which produced maximum stimulation when added to 100 ml. of medium containing hydrolysed purified caseinogen. The dry weights of the various extracts were as follows:

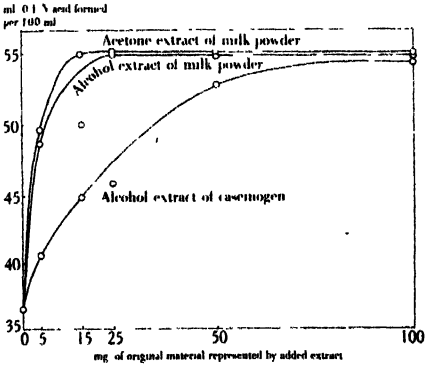

Fig. 3. Stimulating effects of extracts of milk powder and crude caseinogen on acid production.

Extract	Weight of one unit γ	Weight of original material represented by one unit of extract mg.
Alcohol extract of caseinogen	882	100
Alcohol extract of milk powder	555	25
Acetone extract of milk powder	15	15

It should be pointed out that recovery of the factor from the milk powder was probably much lower than from caseinogen, since the latter was extracted continuously whilst the milk powder was merely shaken with the solvents. Milk powder seemed to be the richest and best source of the stimulant, and the acetone extract was by far the most active per unit of dry weight.

Maximum stimulation in 100 ml. of medium was obtained with 0·9 mg. of alcohol extract of caseinogen (the extract of only 0·1 g of crude caseinogen) in the presence of only 0·15 g. hydrolysed purified caseinogen. In contrast to this the optimum concentration of hydrolysed crude caseinogen was 0·9 g. (Fig. 1). This high optimum concentration was probably due to partial destruction of the stimulant during the acid hydrolysis. Enough active stimulant still remained, however, to cause the superiority of hydrolysed crude caseinogen over hydrolysed purified caseinogen.

Response of other cultures to the stimulant.

In order to find out whether cultures of propionic acid bacteria other than *P. pentosaceum* 11 require or are benefited by the factor, five additional cultures were tested on the basal medium with hydrolysed caseinogen (0·15 %) with and without the stimulant. Table I gives the results of this experiment, in which culture 11 is included for comparison. From these results it appears that different strains of propionic acid bacteria vary in their requirements for this alcohol-soluble factor. Cultures 11 and 61 respond very definitely. Cultures 56 and 57 respond to a less degree. Culture 54, a rather weak fermenter, apparently was not affected, whilst culture 33, a vigorous fermenter, grew just as well without the factor. Culture 33 either does not require the stimulant for its growth or, if it does, it can synthesize it under the cultural conditions used.

Table I. *Response of different cultures to the stimulant.**

Addition to basal hydrolysed caseinogen medium	Culture No. 11	33	54	56	57	61
	ml. 0·1 N acid formed per 100 ml. medium					
None	37	53	20	29	32	33
Acetone extract milk powder, 36γ/100 ml.	55	54	21	37	45	62

* Inoculated from second transfer in $(NH_4)_2SO_4$ medium.

Relation of the stimulant to growth stimulants for other organisms.

In order to determine whether our factor was similar to other stimulants described in the literature, as many of these factors as were available were tested for their ability to replace it. The concentration of each substance tested was varied over a wide range on each side of the optimum values reported for other organisms. The figures in Table II give the maximum acid productions with these materials[1] in the basal medium containing 0·15 % hydrolysed purified caseinogen. Inositol, indoleacetic acid, ascorbic acid and nicotinic acid amide had no effect. Pantothenic acid was slightly active in the highest concentration, probably because of impurities present, since 1·25 mg. had less effect than did 15γ of acetone extract of milk powder. The sample was a 50 % pure preparation of the calcium salt of pantothenic acid which according to Dr R. J. Williams stimulated yeast growth in a concentration of 2·9γ per 100 ml.

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[1] The authors wish to thank Dr R. J. Williams for the sample of pantothenic acid and Dr C. J. Koehn for the nicotinic acid amide.

Table II. *Effects of various materials on acid production.*

Material tested	Concentration range per 100 ml.	Activity	
		Concentration* per 100 ml.	Acid formed per 100 ml. ml. 0·1 N
None	—	—	38
Acetone extract of milk powder	0·7 to 70 γ	15·0 γ	55
dl-Inositol	5 to 500 mg.	500 mg.	39
Indoleacetic acid	0·01 to 100 mg.	0·01 mg.	31
Ascorbic acid	0·5 to 100 mg.	50 mg.	41
Nicotinic amide	0·1 to 100 mg.	0·1 mg.	31
Pantothenic acid	0·25 γ to 1·25 mg.	1·25 mg.	47

* Most active or least toxic concentration.

The fractions of liver extract[1] which were tested for stimulation of *L. delbrückii* [Snell *et al.* 1936] were also tested for ability to replace the propionic stimulant. The hepatoflavin fraction was inactive, the vitamin B_2 fraction had some activity, but the ether-alcohol precipitate fraction was quite active. This fraction was also most effective for *L. delbrückii*.

Properties of the stimulant.

In order to obtain some information regarding the chemical nature of the stimulant, various physical properties were investigated. Approximately 5 units of the stimulant were used for each of the various treatments and the preparations and fractions obtained were tested for activity by adding them in varying concentrations to 10 ml. of the basal medium containing 0·15 % purified hydrolysed caseinogen. The results for one concentration (1 unit per 100 ml.) are given in Table III. It should be mentioned that high acidity was accompanied in every case by increased visible cell growth.

Table III. *Physical properties of stimulant.*

Treatment or fraction tested	Acid formed per 100 ml. medium ml. 0·1 N	Conclusion
No stimulant	38	—
Untreated stimulant	55	—
Dried 60°, 12 hours	56	Stable
Heated 1 hour in N H_2SO_4 126°	55	Stable
Heated 1 hour in N NaOH 126°	45	Partially destroyed
Steam distillate, *p*H 4	32	Non-volatile
Steam distillate, *p*H 9	34	Non-volatile
Vacuum distillate, 2 mm. Hg, 120°	39	Non-volatile
Vacuum residue	53	Still active
Acetone extract, *p*H 5·0	53	Extracted
Acetone extract, *p*H 8·0 (NaOH)	57	Extracted
Acetone extract, *p*H 8·0 ($Ba(OH)_2$)	55	Extracted
Chloroform extract, *p*H 4·0	45	Partially extracted
Chloroform extract, *p*H 8·0 ($Ba(OH)_2$)	41	Partially extracted
Ether extract, *p*H 4·0	35	Not extracted
Ether extract, *p*H 8·0	37	Not extracted
Treated $BaSO_4$, *p*H 6·8	56	Not adsorbed
Treated norite, *p*H 6·0	40	Adsorbed
Treated norite, *p*H 8·0	37	Adsorbed
Treated norite, *p*H 7·0	32	Adsorbed

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[1] These extracts were prepared by Dr C. J. Koehn for his vitamin investigations and portions of them kindly supplied to the authors.

The factor is stable to dry heat at a temperature of 60° for 12 hours. It is stable to autoclaving at neutrality and in N H_2SO_4 solution but is largely destroyed by N NaOH under the same conditions. The factor is not volatile with steam either at pH 4 or 9, nor is it volatile at 120° and 2 mm. pressure.

It was hoped that some indication of whether the stimulant was acidic or basic in nature could be obtained by extraction from acid and alkali. Aqueous solutions of the factor were adjusted to pH 4 and 8 with H_2SO_4 and NaOH respectively, dried on anhydrous $CaSO_4$ and extracted continuously with solvents for 24 hours. Acetone extracted the stimulant completely under all conditions, the chloroform extractions were incomplete at both pH levels and the stimulant was not ether-soluble. It seemed possible that the factor might be a neutral compound as there was no difference between recovery from alkaline and from acid solutions.

From the results given in this table it can only be concluded that the factor is stable to acid and labile to alkali, and that it is non-volatile, soluble in acetone, slightly soluble in chloroform, insoluble in ether and adsorbed on norite.

Replacement by vitamin B_1.

The solubilities and stability of the factor suggested that it might be similar to vitamin B_1. Samples of this vitamin were therefore tested for their activity in replacing the stimulant in the basal medium containing 0·15 % hydrolysed purified caseinogen. Fig. 4 shows the effect of these samples as compared with that of acetone extract of milk powder. Both vitamin B_1 samples replaced the stimulant completely. The optimum concentration of acetone extract of milk per 100 ml. was 15γ, that of the I.G. (German) sample of B_1 was 5·0γ and the sample of crystalline B_1 (Merck) was active at a concentration of 0·5γ.

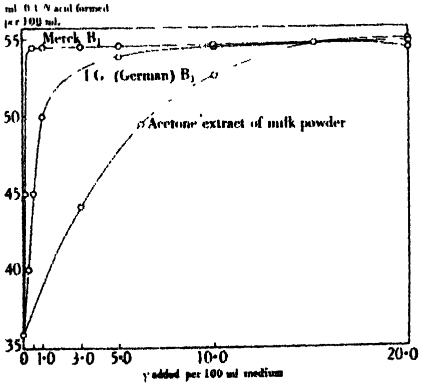

Fig. 4. Effects of vitamin B_1 and acetone extract of milk powder on acid production.

DISCUSSION.

The complete replacement of our stimulant by vitamin B_1 in very low concentrations indicates that the two factors are identical. This supposition is supported by the similarities of the properties of the stimulant and of vitamin B_1. The solubility of the factor in water, alcohol and acetone, the insolubility in ether; the stability to acid and destruction by alkali; the adsorption on norite; and the non-volatility are all characteristic properties of vitamin B_1.

The materials in which our stimulant has been found, yeast, caseinogen and milk, are known to contain this vitamin. Egg albumin and egg white were not as rich in our factor as the other materials and are known to contain little vitamin B_1. The facts that our stimulant is not a part of the protein molecule and that the washed vitamin-free caseinogen was not active further support the theory of its identity with vitamin B_1. It is probable that the activity of vitamin B_1 would not be completely destroyed even by hydrolysis in 20 % H_2SO_4; hence the observed activity of the protein hydrolysates would not contradict the theory that the stimulant is the same as vitamin B_1.

Vitamin B_1 has been reported to be stimulatory or essential for other microorganisms. Schopfer [1935] showed that *Phycomyces blakesleeanus* is stimulated by vitamin B_1 and suggested the use of this organism in vitamin B_1 assay. It has also been reported [Sunderlin & Werkman, 1928] that many bacteria are able to synthesize vitamin B_1. The more vigorous growth of propionic acid bacteria obtained by Sherman & Shaw [1923] in association with certain other bacteria might possibly have been due to synthesis of vitamin B_1 by the associated organisms. *P. freudenreichii* No. 33 may itself synthesize this vitamin, since it grows with equal vigour with or without its addition to the medium.

The function of vitamin B_1 in the metabolism of propionic acid bacteria is not definitely known. Its most important role may be in stimulating growth and multiplication since its most striking effect is the greatly increased cell growth induced. It is of great interest that vitamin B_1, a specialized substance essential for higher animals, is also required by single-celled microorganisms such as the propionic acid bacteria.

SUMMARY.

The stimulating action of protein hydrolysates on the acid production by certain propionic acid bacteria has been found to be due in part to a factor which is neither an amino-acid nor a part of the protein molecule.

The stimulant was obtained from unhydrolysed caseinogen, egg albumin, yeast extract and milk powder by extraction with alcohol or acetone. The acetone extract of milk powder was most effective, since it was active in a concentration of 15 γ per 100 ml.

The effect of the stimulant was most pronounced in the presence of amino-acids and it stimulated four of six cultures tested.

Inositol, pantothenic acid, ascorbic acid, hepatoflavin, nicotinic acid amide and indoleacetic acid could not replace the stimulant.

The properties of the stimulant were similar to those of vitamin B_1, and crystalline vitamin B_1 (Merck) completely replaced it in a concentration of 0·5 γ per 100 ml. of medium.

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CCLXVIII. THE OCCURRENCE AND POSSIBLE SIGNIFICANCE OF SOME OF THE MINOR COMPONENT ACIDS OF COW MILK FAT.

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The occurrence in butter fatty acids of minute proportions (about 0·2 %) of Δ^9-decenoic acid, first reported by Smedley [1912], has been definitely established by Grün & Wirth [1922] and by Bosworth & Brown [1933]. Grün & Winkler [1924] mentioned in addition the presence of small quantities of do-, tetra- and hexa-decenoic acids, but Bosworth & Brown, who showed that a tetradecenoic acid forms nearly 1 % of the total butter fatty acids, were unable to detect the presence of hexa- or do-decenoic acids. Riemenschneider & Ellis [1936], however, have recently reported that goat milk fat contains 2·1 % of hexadecenoic acid, as well as smaller amounts of tetradecenoic (0·4 %) and decenoic (0·2 %) acids. No information appears to have been given as to the structure of the tetra- and hexa-decenoic acids of these milk fats. In a recent communication [Hilditch & Thompson, 1936] we described oxidation experiments conducted on certain of the lower fractions obtained from the esters of the "liquid" fatty acids of butter during ester-fractionation analysis, which showed that the above-mentioned lower unsaturated acids were clearly detectable in the ester fractions in question, and which indicated the presence of hexadecenoic acid as definitely as that of tetradecenoic acid.

We have now given this question some further study, primarily in order to ascertain how far the quantitative data for the major component acids, as determined by our ester-fractionation procedure, are affected if (as hitherto) no account is taken of the minor quantities of lower unsaturated acids present. Concurrently, we have endeavoured to ascertain whether any unsaturated acid of lower molecular weight than decenoic acid is present, and also to obtain evidence as to the position of the unsaturated group in the tetra- and hexa-decenoic acids, the presence of both of which is indicated by quantitative oxidation of appropriate ester fractions (*v. infra*).

For this purpose we have utilized the ester fractions obtained during the analysis, for Prof. Kay of the National Institute for Research in Dairying, of the component acids of the milk fat of a cow which had been receiving daily injections of 10 mg. of thyroxine. The composition of this milk fat was entirely normal and similar to those which we have previously examined from cows on normal diet; we therefore considered that the treatment of the cow constituted no objection to use of the material from this analysis for the additional experiments. We applied our usual ester-fractionation procedure to the butter fatty acids subject, however, to the following modifications:

(*a*) After the fatty acids volatile in steam had been fractionally distilled, the I.V., as well as the equivalent, of each fraction was determined.

(*b*) The esters obtained from the "liquid" acids (resulting from the lead salt-alcohol separation of the acids non-volatile in steam) were divided into two portions. One portion was distilled in our usual way, a series of small fractions being collected from the refractionation of the first primary fraction; with the other portion, the size of the fractions was modified to some extent, in the hope of collecting larger quantities of those containing methyl tetra- or hexa-decenoate.

(c) In addition to the foregoing analysis, we carried out an ester-fractionation analysis on a specimen of the butter fat which had been completely hydrogenated, the results of which lead to the percentage composition of the mixed, completely saturated, fatty acids and which thus avoid any uncertainty due to the presence of small proportions of unsaturated acids other than those of the C_{18} series.

The cow milk fat studied had the following general characteristics: Reichert-Meissl value 27·3, Polenske value 2·1, Kirschner value 23·2, sap. equiv. 247·8 and I.V. 41·0.

Ester-fractionation analyses of the mixed fatty acids.

The fat (489·5 g.) yielded, after hydrolysis and prolonged distillation in steam of the resulting fatty acids, 435·8 g. of acids non-volatile in steam, while from the aqueous condensates there were ultimately recovered 29·7 g. of steam-volatile fatty acids. The acids non-volatile in steam were submitted to the usual lead salt separation from alcohol with the following result:

		Corresponding methyl esters	
	%	Equiv.	I.V.
"Solid" acids	44·2	274·3	4·7
"Liquid" acids	55·8	272·7	76·0

Fractionation of the fatty acids volatile in steam.

The aqueous condensates from the steam-distillation were extracted exhaustively with ether and the dried recovered acids submitted to direct fractional distillation, with the results given in Table I.

Table I. *Fractional distillation of acids volatile in steam.*

	g.	Equiv.	I.V.
Acidity (as butyric acid) in:			
Ether-extracted aqueous solution	1·14	—	—
Recovered ether	0·36	—	—
Distillate fractions:			
1 (up to 100°)	0·13	—	—
2 (100–160°)	1·53	—	—
3	2·24	90·0	0·0
4	2·70	90·6	0·0
5	2·60	91·7	0·0
6	2·45	92·0	0·0
7	2·78	93·7	0·0
8	2·58	95·9	0·1
9	3·72	103·7	0·1
10	2·71	115·8	0·2
11	3·05	129·3	1·7
Residue	1·68	180·2	17·9

The fairly substantial I.V. of the residues from this particular distillation have been calculated to oleic acid in our previous papers, in consequence of the observation [Hilditch & Sleightholme, 1930] that pure oleic acid is volatile in steam to about the same extent as that indicated by the I.V. in question. Calculated as oleic acid, I.V. 17·9 corresponds to 0·33 g., or 0·07 % oleic acid in the total fatty acids; reckoned as decenoic acid, it amounts to 0·20 g., or 0·043 %

decenoic acid in the total fatty acids. The proportion of unsaturated acid involved in these residues is thus so small that the final composition of the total fatty acids is not affected to more than 0·03 % by assuming it to be either decenoic or oleic.

Only the final fraction of distillate had any appreciable I.V. (1·7), representing 0·03 g. as decenoic acid (less than 0·01 % of the total fatty acids); the mean equivalent of this fraction lies between those of hexanoic and octanoic acids, and the 1 % of unsaturated acid present therein may well be decenoic, although of course it might conceivably be traces of an octenoic acid. Clearly, however, there is no sign of the presence of octenoic or lower unsaturated acids in amounts approaching even the small proportions (0·2 %) in which decenoic acid has been recorded in milk fats.

Fractionation of methyl esters of the "solid" and "liquid" acids.

As already mentioned, two separate fractionations of the methyl esters of the "liquid" acids were undertaken, the second being carried out in an

Table 11. *Fractional distillation of methyl esters.*

(*a*) *Methyl esters of "solid" acids.*

Primary fractionation				Refractionation of fraction S1			
No.	g.	Sap. equiv.	I.V.	No.	g.	Sap. equiv.	I.V.
S1	31·52	261·5	1·7	S11	2·43	244·9	1·0
S2	6·24	272·3	2·7	S12	3·09	250·8	1·1
S3	8·21	273·7	3·2	S13	4·05	254·3	1·1
S4	8·11	277·4	4·1	S14	6·17	261·6	1·4
S5	7·75	280·7	5·8	S15	7·22	271·8	2·6
S6	8·26	287·6	8·8	S16	5·01	278·3	6·4
S7	9·22	294·9	10·9		27·97		
S8	3·28	304·6	12·9				
	82·59						

(*b*) *Methyl esters of "liquid" acids.*

Analysis I.

Primary fractionation				Refractionation of fractions L1			
No.	g.	Sap. equiv.	I.V.	No.	g.	Sap. equiv.	I.V.
1 L1	31·83	232·7	39·4	1 L11	2·30	171·3	5·0
1 L2	19·15	286·1	84·3	1 L12	2·19	187·9	8·8
1 L3	10·53	291·1	90·9	1 L13	2·80	199·5	9·3
1 L4	8·84	291·5	92·9	1 L14	12·79	241·3	29·1
1 L5	8·53	293·9	94·3	1 L15	4·68	279·3	74·5
1 L6	8·38	294·6	95·8	1 L16	3·66	293·4	89·2
1 L7	9·50	294·7	96·2		28·42		
1 L8	5·41	324·3	105·2				
	102·17	300·7*	99·9*				

Analysis II.

No.	g.	Sap. equiv.	I.V.	No.	g.	Sap. equiv.	I.V.
2 L1	68·60	251·3	56·8	2 L11	7·11	180·6	9·1
2 L2	8·77	291·0	84·7	2 L12	7·25	214·7	12·1
2 L3	10·53	293·6	92·7	2 L13	15·15	248·7	36·2
2 L4	9·86	294·4	94·2	2 L14	20·51	281·2	78·3
2 L5	9·14	294·8	94·9	2 L15	14·78	292·7	90·8
2 L6	10·67	296·0	96·8		64·80		
2 L7	4·97	295·9	99·0				
2 L8	4·16	346·0	112·4				
	126·70	297·4*	100·0*				

* Residual esters, freed from unsaponifiable.

attempt to obtain larger quantities of certain of the fractions in view of their subsequent oxidation (to determine the mean equivalent of the saturated esters present). The details of all the fractional distillations are collected in Table II.

In order to obtain data for the calculation of mixtures of two unsaturated esters (e.g. tetra- and hexa-, or hexa- and octa-decenoates) along with saturated esters in the "liquid" ester fractions, some of the latter were oxidized in acetone solution with powdered $KMnO_4$; the saturated esters were thereby isolated and their equivalent determined. (It is difficult to ensure quantitative recovery of the purified saturated esters and it is therefore preferred to employ only their equivalent in the calculation. The equivalent and I.V. of the original ester fraction, with the mean equivalent of the saturated components therein, afford the data necessary for the calculation of the weights of two saturated and two unsaturated components.) The results of these oxidation analyses are given in Table III.

Table III. *Saturated esters present in certain "L" fractions (Table II).*

Ester fraction				Saturated ester
No.	g.	Sap. equiv.	I.V.	Sap. equiv.
1 L14	12·79	241·3	29·1	237·5
1 L2	19·15	286·1	84·3	265·4
2 L12	7·25	214·7	12·1	221·4
2 L13	15·15	248·7	36·2	243·2
2 L14	20·51	281·2	78·3	264·0

Loss of some of the more volatile saturated esters prevented the determination of the mean equivalent of the saturated components in fraction 2 L11, and it is possible that the value 221·4 found for those in fraction 2 L12 is somewhat higher than the true figure for the same reason. In the case of 2 L13, sufficient saturated esters (6·92 g. from 12·51 g. oxidized) were recovered to permit their further resolution into three fractions by distillation (2·06, 2·16, 2·02 g. with equivalents respectively 234·5, 244·5 and 251·0). The composition of the saturated methyl esters in 2 L13 was thus laurate 8·0 %, myristate 77·4 % and palmitate 14·6 %.

We have calculated the composition of the total fatty acids of the milk fat in three ways: (*a*) calculating all unsaturation as due to C_{18} unsaturated acids in the manner hitherto adopted and (*b*) allowing for decenoic, tetra- and hexa-decenoic acids as indicated by the oxidation data in Table III for each of the fractionations of "liquid" methyl esters numbered I and II in Table II. As pointed out in a previous paper [Hilditch & Thompson, 1936], a slight error is necessarily introduced in the latter case, because a little methyl oleate distils over in all the fractions, and the fractionation procedure is unable to yield a precise estimate of the amounts of the minor unsaturated acids present. Nevertheless the results obtained for decenoic and tetradecenoic acids are of the order given by Bosworth & Brown as a result of their study of several kg. of butter fatty acids and are further of interest when compared with the figures for the hydrogenated butter fatty acids which are given later in this paper. For the purposes of calculation the fractions in the analyses I and II have been computed as follows.

1 L11, 1 L12, 1 L13	Saturated esters + methyl decenoate
1 L14	Saturated esters (equiv. 237·5) + methyl tetra- and hexadecenoates
1 L15, 1 L2...	Saturated esters (equiv. 265·4) + methyl hexadecenoate and C_{18} unsaturated esters
1 L16, 1 L3, 1 L4, 1 L5, 1 L6, 1 L7	Methyl palmitate + hexadecenoate + C_{18} unsaturated esters
2 L11, 2 L12	Saturated esters + methyl decenoate
2 L13	Saturated esters (equiv. 243·2) + methyl tetra- and hexadecenoate
2 L14	Saturated esters (equiv. 264·0) + methyl hexadecenoate and C_{18} unsaturated esters
2 L15, L2, L3, L4, L5	Methyl palmitate + hexadecenoate + C_{18} unsaturated esters

The final results (weight and molar percentages) for the three alternative calculations are given in Table IV.

Table IV. *Component acids of cow milk fat.*

(*a*) Without allowance for unsaturated acids below C_{18}.
I Utilizing oxidation data (as above) from analysis I (Tables II and III).
II Utilizing oxidation data (as above) from analysis II (Tables II and III).

	Weight percentages			Molar percentages		
Acid	(*a*)	I	II	(*a*)	I	II
Butyric	3·7	3·7	3·7	9·8	9·8	9·7
n-Hexanoic	2·0	2·0	2·0	4·1	4·1	4·2
n-Octanoic	1·3	1·0	0·9	2·1	1·6	1·5
n-Decanoic	2·7	2·6	2·6	3·7	3·5	3·5
Lauric	4·0	1·7	2·7	4·8	2·0	3·2
Myristic	7·9	9·3	8·6	8·2	9·6	8·9
Palmitic	23·8	25·4	26·2	21·9	23·4	24·0
Stearic	10·7	10·7	10·7	8·9	8·9	8·8
As arachidic	0·5	0·4	0·5	0·3	0·3	0·3
As decenoic	—	0·2	0·5	—	0·3	0·6
As tetradecenoic	—	1·2	0·7	—	1·3	0·8
As hexadecenoic	—	5·0	4·9	—	4·6	4·5
As oleic	38·3	32·4	31·9	31·9	27·0	26·5
As octadecadienoic	4·7	4·0	3·9	4·0	3·3	3·3
As C_{20-22} unsaturated	0·4	0·4	0·2	0·3	0·3	0·2

The results of these alternative calculations are very similar to those for two other cow milk fats studied similarly by Hilditch & Thompson [1936]. They point to the presence of decenoic and tetradecenoic acids in proportions closely similar to those suggested by Bosworth & Brown, but they also indicate the presence of more hexadecenoic acid than of either of the former. The effect of allowance for these minor proportions of lower unsaturated acids upon the major component acid figures is to reduce the oleic acid percentage by about 5 % (mol.) and to increase the palmitic acid by about 2 % (mol.); the rest of the acids are not significantly affected.

Ester-analysis of the completely hydrogenated butter fat.

Further evidence in regard to the proportions of total C_{14}, C_{16} and C_{18} acids present in the cow milk fat has been obtained by carrying out a fractionation analysis on the acids from the fat after it had been completely hydrogenated.

The butter fat (400 g.), without any previous refining treatment, absorbed hydrogen in presence of nickel on kieselguhr at 180° with great ease, yielding a white solid fat of I.V. 0·1. The hydrogenated fat (301·9 g.) yielded 16·1 g. of acids volatile in steam and 270·6 g. of acids non-volatile in steam. The details of the fractionation analyses for each group of acids are given in Tables V and VI.

Table V. *Fractional distillation of acids volatile in steam (hydrogenated butter fat).*

		g.	Equiv.
Acidity (as butyric acid) in:			
	Ether-extracted aqueous solution	1·03	—
	Recovered ether	0·12	—
Distillate fractions:	1 (up to 100°)	1·46	—
	2 (100–160°)	2·83	—
	3	2·57	93·0
	4	2·06	94·3
	5	2·06	100·1
	6	1·94	115·6
	7	0·99	129·5
	8	1·06	170·5

Table VI. *Fractional distillation of methyl esters of acids non-volatile in steam (hydrogenated butter fat).*

Primary fractionation			Refractionations		
No.	g.	Sap. equiv.	No.	g.	Sap. equiv.
1	28·98	235·7	11	1·70	174·6
			12	2·30	193·4
			13	2·65	214·4
			14	3·24	232·5
			15	3·49	245·0
			16	3·98	253·4
			17	4·66	265·2
			18	3·96	281·6
				25·98	
2	41·16	276·4	21	4·06	265·0
			22	4·05	267·6
			23	4·58	270·8
			24	4·17	273·5
			25	4·12	275·5
			26	3·87	280·2
			27	4·03	286·0
			28	2·70	290·3
			29	3·84	295·2
				35·42	
3	6·25	285·2			
4	7·34	288·5			
5	7·34	291·8			
6	6·26	293·8			
7	6·87	295·9			
8	6·71	296·5			
9	5·43	298·7			
10	2·90	299·2			
11	4·92	321·7	(Residual esters, freed from unsaponifiable, sap. equiv. 303·5)		
	124·16				

These figures lead to the following composition for the total acids in the hydrogenated butter fat:[1]

Acid	% (wt.)	% (mol.)
Butyric	3·5	9·4
n-Hexanoic	1·6	3·2
n-Octanoic	0·7	1·2
n-Decanoic	2·6	3·6
Lauric	3·6	4·3
Myristic	7·9	8·2
Palmitic	34·0	31·6
Stearic	45·2	37·9
As C_{20-22} saturated	0·9	0·6

The molar percentages of each group of acids (C_{10}, C_{14}, C_{16}, C_{18}) from the analyses of the original butter fatty acids given in Table IV, (*a*) without and (I and II) with allowance for lower unsaturated acids, may now be compared with the above results from the hydrogenated butter fat (Table VII):

Table VII. *Molar percentages of the different fatty acid groups*

From analysis of ...	Original butter fat			Hydrogenated butter fat
Acids	(*a*)	I	II	
C_4	9·8	9·8	9·7	9·4
C_6	4·1	4·1	4·2	3·2
C_8	2·1	1·6	1·5	1·2
C_{10}	3·7	3·8	4·1	3·6
C_{12}	4·8	2·0	3·2	4·3
C_{14}	8·2	10·9	9·7	8·2
C_{16}	21·9	28·0	28·5	31·6
C_{18}	44·8	39·2	38·6	37·9
C_{20-22}	0·6	0·6	0·5	0·6

It is evident that columns I and II (i.e. with allowance for the lower unsaturated acids) are in much better agreement with the data for the hydrogenated butter fat than column (*a*). The C_{16} acid content of the hydrogenated butter fat is indeed higher than that as calculated under I and II, which would point to the presence of even more hexadecenoic acid than our figures suggest. We are aware however that the separation of methyl myristate from a mixture containing large proportions of methyl stearate as well as methyl palmitate is difficult and it is possible that, in spite of the refractionation of fraction 2 (in Table VI), this separation has not been quite complete. It is therefore possible that the values in the final column for C_{14} and C_{18} acids may be slightly below, and that for C_{16} acid correspondingly above, the true figures.

The values for the component acids of the hydrogenated fat, at all events, definitely confirm the presence of hexadecenoic acid and, on the whole, support the modified calculations I and II which we have employed in order to allow for the presence of this and the other two lower unsaturated acids (C_{10} and C_{14}) in the original milk fat.

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[1] We have satisfactorily verified the identity of myristic and palmitic acids in some of the fractions given in Table VI, but we have not succeeded in definitely establishing the presence of lauric acid. The acids from fraction 13, for example, which might have been rich in methyl laurate, failed to yield an individual specimen of lauric acid on repeated recrystallization; crystallization of the anilides prepared from the acids of this fraction also failed to reveal the presence of lauranilide. We are consequently doubtful whether lauric acid is actually present in milk fats, even in the small proportions suggested.

Structure of tetra- and hexa-decenoic acids of butter fat.

Although the position of the double bond in the 0·2 % of decenoic acid present in butter fat has been given ($\Delta^{9:10}$) by Grün and by Bosworth & Brown, the structures of the tetra- and hexa-decenoic acids have not been stated. We have attempted to obtain evidence on this point by examining the mono- and dicarboxylic acids produced in the course of $KMnO_4$-acetone oxidation of fraction 2 L13 (Table II), in which methyl tetra- and hexa-decenoate were both present. The esters (12·8 g.), after oxidation in acetone with powdered $KMnO_4$, removal of the acetone and decoloration of the manganese oxides, were extracted with ether from the aqueous salt solution. The ether extract was washed repeatedly with aqueous K_2CO_3 and the united aqueous extracts were heated with aqueous KOH in order to hydrolyse the monomethyl esters of dicarboxylic acids produced during the oxidation. The solution was finally concentrated to a small bulk, made acid and distilled for some hours in steam in order to separate the mono- from the di-carboxylic acids.

The residual liquor from the steam-distillation was repeatedly extracted with ether and yielded 2·87 g. of solid acids. These were refluxed with petroleum (B.P. 40–60°) to remove any remaining monobasic acids. The undissolved acids were recrystallized from water; they melted at 98–100° and had an equivalent of 96·5; on further crystallization from chloroform the melting-point was raised to 105° (unchanged when mixed with azelaic acid), the equivalent then being 93·7 (azelaic acid, 94·0). The mother-liquors from the various crystallizations were examined for other dicarboxylic acids, but without result. The yield of crude azelaic acid (2·87 g.) from the 4·7 g. of unsaturated methyl esters present in the specimen oxidized corresponds to 61 % of the weight of the unsaturated esters and suggests that in all the latter the unsaturation was in the $\Delta^{9:10}$-position. (The theoretical yields of azelaic acid from methyl $\Delta^{9:10}$-tetradecenoate, $\Delta^{9:10}$-hexadecenoate and $\Delta^{9:10}$-octadecenoate are respectively 78, 70 and 63 %; the yield of azelaic acid actually isolated from a $\Delta^{9:10}$-unsaturated ester by the $KMnO_4$-acetone method is about 85–90 % of theory [Armstrong & Hilditch, 1925].)

The aqueous condensates from the steam-distillation were extracted with ether and worked up as in the case of the steam-volatile acids from a butter analysis. The ether-extracted aqueous washings still contained water-soluble acids equivalent to 24·1 ml. of $N/10$ alkali, the nature of which was not determined. The acids extracted by ether gave, on distillation, a fraction of equivalent 127·8 and a subsequent smaller fraction with equivalent 141, pointing to the presence of *n*-heptanoic acid (equivalent 130) accompanied by small quantities of acids of lower and higher mol. wt.

Evidence was thus afforded of the presence of *n*-heptanoic and azelaic acids (corresponding with the oxidation products of $\Delta^{9:10}$-hexadecenoic acid), while in addition no other dicarboxylic acid was detected and the total yield of azelaic acid was of the order which would be encountered if in all the unsaturated acids present the double bond was in the $\Delta^{9:10}$-position. It therefore seems that the position of the ethenoid bond, in the three lower unsaturated acids which form minor components of the butter glycerides, is the same with respect to the carboxylic group as in oleic acid:

$CH_2:CH.[CH_2]_7.COOH$	Decenoic acid
$CH_3.[CH_2]_3.CH:CH.[CH_2]_7.COOH$	Tetradecenoic acid
$CH_3.[CH_2]_5.CH:CH.[CH_2]_7.COOH$	Hexadecenoic acid
$CH_3.[CH_2]_7.CH:CH.[CH_2]_7.COOH$	Oleic acid

The relatively high proportions of fully-saturated glycerides in animal milk and depot fats have been pointed out in former communications from this laboratory and it has been suggested that stearo-glycerides in depot fats may result by saturation of pre-formed oleo-glycerides [Banks & Hilditch, 1931; 1932], and that the lower saturated acids combined as glycerides in milk fats may also result from transformation of an oleo-glyceride into, for example, a butyro- or a myristo-, etc. glyceride [Hilditch & Sleightholme, 1931]. The latter view, involving chemical change commencing from the end of the alkyl fatty acid chain instead of from the carboxylic group, is perhaps now not so unconventional as when it was first put forward, owing to the recent observations of Jowett & Quastel [1935] on "multiple alternate oxidation" as opposed to the simple theory of β-oxidation, and of Verkade & van der Lee [1934] on ω-oxidation of fats. The identity of the position of the double bond in the minor lower unsaturated acids of butter fat with that in oleic acid, with the apparent absence of any unsaturated acid below $\Delta^{9:10}$-decenoic acid, is also in harmony with this conception of milk fat formation, for the acids in question may represent fragments, so to speak, of transformed oleo-glycerides which have escaped final saturation to lower saturated glycerides.

SUMMARY.

Detailed analyses have been made of the component acids of a typical cow milk fat, and the results calculated (*a*) on the assumption that no unsaturated acids of lower molecular weight than oleic were present and (*b*) allowing for the presence of decenoic, tetra- and hexa-decenoic acids on the basis of special analyses to determine the saturated ester contents of certain of the ester fractions obtained in the course of the fractional distillations of "liquid" methyl esters. Comparison of these results with those for the similar analysis of the component acids of the completely hydrogenated butter shows that the total proportions of C_{16} and C_{18} acids present are in agreement only when allowance has been made, as in (*b*), for the presence of lower unsaturated acids.

In addition to the traces of decenoic acid and somewhat over 1 % of tetradecenoic acid reported by Bosworth & Brown, there must be about 4–5 % of hexadecenoic acid also present. As regards the chief component acids, palmitic and oleic, the result of the correction for the lower unsaturated acids is to increase the palmitic acid figure by about 2 %, while the oleic acid figure is reduced by about 5 %.

There is no detectable amount of any unsaturated acid of lower molecular weight than $\Delta^{9:10}$-decenoic acid in butter fat, and the positions of the double bond, relative to the carboxyl group, are the same in the decenoic, tetra-, hexa- and octa-decenoic acids of butter fat. It is suggested that these observations are in harmony with the hypothesis put forward in earlier communications, namely, that the lower saturated glycerides of milk fats have been produced from preformed oleo-glycerides, and that these minor, lower unsaturated components may represent degradation products of oleo-glycerides which have escaped complete saturation to lower saturated groups.

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CCLXIX. THE ACTION OF PHENYL *ISO*CYANATE ON INSULIN.

II. FURTHER OBSERVATIONS ON THE CHEMISTRY OF INSULIN AND ITS PHOSPHATE-LOWERING POWER.

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Hopkins & Wormall [1934, 2] found that the action of phenyl *iso*cyanate on insulin caused complete, or almost complete, loss of hypoglycaemic activity very rapidly at *p*H 8 and at 5–8°. Previous chemical and immunological investigations on various phenylcarbamido-protein derivatives [Hopkins & Wormall, 1933, 1, 2; 1934, 1] indicate that phenyl *iso*cyanate probably reacts with free amino-groups and causes no other very drastic change in the protein molecule. The conclusion was reached, therefore, that the free amino-groups of insulin, or at least some of them, are essential for its activity. Jensen *et al.* [1934] had simultaneously reached the same conclusion [cf. also Jensen & Evans, 1935].

Various authors have recorded other changes in the blood of animals following insulin injection. Thus Wigglesworth *et al.* [1923] observed a fall in the blood inorganic phosphate of rabbits, and Briggs *et al.* [1923–24] found a decrease in the blood glucose, potassium and inorganic phosphate and a rise in the blood lactic acid of dogs. Luck *et al.* [1928] have recorded a fall in the blood amino-acid, and other authors have found increases in blood lactic acid and calcium [cf. Peters & Van Slyke, 1931]. Rigo & Frey [1934] found a diminution in blood creatine and creatinine after insulin injection.

Some of these changes, e.g. those in lactic acid and phosphate and, less directly, calcium, appear to be closely connected with the fall in the blood sugar level. (A review of the literature dealing with the relationship between phosphates and carbohydrate metabolism is given by Peters & Van Slyke [1931, pp. 1114–1118], and more recent work is discussed by Cori & Cori [1934, p. 165].)

From this and other evidence it seems probable that the hypophosphataemic capacity of insulin is secondary to its influence on carbohydrate metabolism, and that insulin has no specific direct action on the blood phosphate. Davis *et al.* [1933], however, have recorded that the phosphate-lowering activity of insulin was more resistant to acid alcohol and soft X-radiation than was the sugar-lowering power, and they conclude that insulin owes its activity to a number of "active groups" which vary in stability towards inactivating agents. Savino [1924] found that when glucose is injected to maintain the normal blood sugar level in a fasting sheep after the injection of insulin, there is still a fall in the blood inorganic phosphate.

We have ourselves carried out experiments to determine whether this differentiation could be effected by some other inactivating agent, since confirmation of the results of Davis *et al.* [1933], by some other method, would demonstrate fairly conclusively the independence of these two activities of insulin. For this purpose, further use has been made of the inactivation by phenyl *iso*cyanate, this reagent being most suitable in view of the mild conditions of the reaction and the relatively specific action of the reagent for one type of grouping.

The action of phenyl *iso*cyanate on insulin and other proteins has been investigated further from the chemical standpoint. In particular, the possibility of a reaction with the hydroxyl group of tyrosine, or with basic groups other than the α-amino-groups of terminal amino-acids and the ε-amino-groups of lysine molecules, has been considered. Where the results of these other investigations are applicable to insulin they are discussed below.

EXPERIMENTAL.

In most experiments the inactivation of insulin by phenyl *iso*cyanate and the determination of the hypoglycaemic actions of treated and untreated insulin were carried out as described previously [Hopkins & Wormall, 1934, 2]. Rabbits which had been starved for 24–36 hours were used for all the assays, and the blood inorganic phosphate determinations were made by the method of Fiske & Subbarow [1925]; 0·25 % phenol was generally added to the insulin and NaCl solutions injected. The volume of solution injected into each rabbit was 0·5 ml. per kg.

The insulin was supplied by Messrs Boots Pure Drug Co. Ltd., and had an activity of 19,500 units per g.

Normal fluctuations in the blood inorganic phosphate of rabbits.

In many experiments there appeared to be significant diurnal fluctuations of the blood inorganic phosphate; the level generally rose during the experiment, with a partial return to the original level later in the day and a more or less complete return in 24 hours (cf. Table I).

Table I. *Fluctuations in the blood inorganic phosphate of rabbits.*

(mg. P per 100 ml. blood.)

		Time after first bleeding (hours)					
	Rabbit	0	2	4½	24	28	29½
Exp. 1	S 7	2·01	2·27	2·60	2·44	—	3·44
	S 8	2·77	2·66	2·98	3·16	—	3·88
	C	2·68	3·13	3·03	2·65	—	3·31
	D	2·79	3·19	3·08	2·60	—	3·59
	Average	2·56	2·81	2·92	2·71	—	3·55
Exp. 2	Average for 8 rabbits	2·92	3·17	3·38	3·16	3·42	—

During these experiments there was no significant alteration in the blood sugar level, and it has not been found possible as yet to account fully for the fluctuations in the phosphate. As the following experiment will indicate, they do not appear to be related to the water intake of the rabbits during the experiment. One group of four rabbits was allowed water *ad lib.* for 2 days before, and during, the 6 hours of the experiment and another group was given no water during the whole of this period. The water consumed by the rabbits in the first group varied from practically nothing to a considerable volume, but all the rabbits showed very similar fluctuations throughout the day. The blood inorganic phosphate figures for two experiments were as follows (average values, as mg. P per 100 ml. blood, for each group):

	Time (hours) ...	0	3	6
Exp. 1	Group A	3·67	4·24	3·58
	Group B	2·82	3·28	2·75
Exp. 2	Group A	2·67	2·77	2·80
	Group B	3·16	3·43	3·24

Group A, given water. Group B, given no water.

From these figures, and from those of other experiments, the water intake does not appear to influence the blood inorganic phosphate, but it must be emphasized that the rise does not invariably occur. Thus in some experiments only a very slight rise, or even none at all, took place, as will be seen from the second experiment mentioned above and from one of the experiments described below (cf. Table III).

These normal fluctuations must, of course, be considered when the effect of insulin on the blood inorganic phosphate is studied, and in all the investigations reported here a control group of rabbits fed on the same diet and under exactly the same experimental conditions has been examined. In connexion with this fluctuation in the blood phosphate it is of interest to note that Havard & Reay [1925] found that there is often, but not invariably, a rise in the blood inorganic phosphate of man during the day, and on one occasion the midday value was 16 % above the morning level. No satisfactory explanation for these variations could be advanced by these authors. In our experiments it seems possible that the withdrawal of 4 ml. of blood from each rabbit at each bleeding might significantly affect the inorganic phosphate level of the blood, but this explanation would not account for the rise observed by Havard and Reay, who withdrew each time less than 1 ml. of blood from the subject. Some observations on the influence of haemorrhage on the blood calcium of rabbits are given by Culhane [1927; 1930] who noted a fall with most of the animals, and it is not inconceivable that this fall in calcium and the rise in inorganic phosphate are in some way interrelated.

The phosphate-lowering power of the phenylcarbamido-derivative of insulin.

A solution of insulin in dilute $NaHCO_3$ and cooled to 5–8° was treated with small amounts of phenyl *iso*cyanate, and the reaction maintained at *p*H 8–8·5 as previously recorded [Hopkins & Wormall, 1934, 2]. The whole mixture was

Table II. *Influence of phenyl* iso*cyanate on the hypophosphataemic power of insulin.*

(Average values for groups of 4 rabbits—results as mg. inorg. P or sugar per 100 ml. of blood.)

	Control		Insulin (0·75 unit per kg.)		Phenylcarbamido-insulin (15 units per kg.)	
	Time (hours)	Inorg. P	Inorg. P	Sugar	Inorg. P	Sugar
Exp. 1	0	2·82	2·85	97	2·65	101
	2	3·38	2·44	45	3·21	108
	4½	3·38	2·66	—	3·09	—
	24	2·98	3·00	—	2·96	—
	28	3·36	3·66	—	3·31	—
Exp. 2	0	3·22	2·91	102	3·43	90
	3½	3·25	2·25	53	3·52	95
	6	3·29	2·48	71	3·58	112

kept overnight, and was then tested, together with untreated insulin, for hypoglycaemic and hypophosphataemic actions in starved rabbits. The treated insulin was used in amounts equivalent to twenty or thirty times the quantity of unchanged insulin normally used for standardization tests. The experiments made under different conditions and with varying amounts of the treated insulin, gave similar results, and all indicated that where loss of the hypoglycaemic activity occurred, there was also loss of hypophosphataemic activity (Table II). As in previous experiments the control animals received injections of a suspension of diphenylurea, a substance which is present in relatively large amounts in the preparations of phenylcarbamido-derivative of insulin used.

Further experiments have been carried out to determine whether it is possible, by using a smaller amount of phenyl *iso*cyanate or by stopping the reaction at different stages, to distinguish between the hypoglycaemic and hypophosphataemic activities of insulin. Table III records an experiment of this type, in which the amount of *iso*cyanate used was less than previously and in addition the reaction was stopped at certain intervals, as far as this was possible, by extraction with ether to remove the excess of phenyl *iso*cyanate. The results indicated a rough parallelism between the two activities of insulin, and there was no evidence of any preferential destruction of the hypoglycaemic or hypophosphataemic power. In several of these experiments it has been noticed that the injection of phenylcarbamido- or *p*-bromophenylcarbamido-derivatives of insulin has been followed by an unexpected slight rise in the blood sugar, whereas the control animals did not show this phenomenon. This rise may be due to some indirect action of the phenylcarbamido-derivative or to the presence in the original insulin of a hyperglycaemic principle [cf. Bürger & Kramer 1930; Dirscherl, 1931]. This hyperglycaemic substance appears to be more resistant to phenyl *iso*cyanate than is the hypoglycaemic hormone.

Table III. *Influence of insulin, which has been partially inactivated by phenyl* iso*cyanate, on the blood inorganic phosphate of starved rabbits.*

(Average values for groups of 4 rabbits.)

				Phenylcarbamido-insulin	
	Time after injection (hours)	Control	Insulin (0·75 unit per kg.)	1st product (5 units per kg.)	2nd product (15 units per kg.)
Blood inorg. P (mg. per 100 ml.)	0	4·05	3·02	2·68	2·79
	2	4·06	2·32	2·07	2·16
	4½	4·23	2·90	2·24	2·57
Blood sugar (mg. per 100 ml.)	0	—	106	100	96
	2	—	59	67	49
	4½	—	45	58	48

Experimental details. 32·8 mg. insulin (640 units) were dissolved in 4 ml. of 0·9 % NaCl plus 4 ml. of 0·2 *M* $NaHCO_3$. 2 *N* NaOH was added to give *p*H 8–8·5, and the solution cooled in ice. After a sample (2 ml.) had been withdrawn, 0·08 ml. of phenyl *iso*cyanate was added to the main bulk and the mixture shaken. Another 2 ml. were taken immediately ("1st product" in the above table), and the remainder was shaken well for 15 min., the solution being kept cool and maintained at *p*H 8–8·5 ("2nd product").

Immediately after it had been taken, each sample was treated with 0·05 ml. of 2 *N* HCl (double quantity for the last sample) and at once extracted with ether to remove the excess of phenyl *iso*cyanate. Six successive extractions with 10 ml. of ether at each extraction were made, and after the addition of 0·05 ml. of 2 *N* NaOH the aqueous solution was freed from ether by gentle warming and evacuation. These solutions were then adjusted to about *p*H 8, and before being used for injection, phenol was added to them to the extent of 0·25 %.

A chemical study of the groups in insulin which might react with phenyl isocyanate.

In previous work [Hopkins & Wormall, 1933, 1; 1934, 1] evidence has been adduced that the main reaction between phenyl *iso*cyanate and proteins is that between the *iso*cyanate and the free amino-groups. With native proteins the reaction is possibly confined to the ε-amino-groups of the lysine molecules, but with proteins of the peptone type, whose phenylcarbamido-derivatives were investigated by Raper [1907], or with insulin, the lysine groupings appear to account for a fraction only of the total free amino-nitrogen. From the chemical viewpoint it seems of importance therefore to study (*a*) the free amino-groups of the insulin molecule which are attacked by phenyl *iso*cyanate, i.e. to find out if possible the amino-acids in insulin which have free amino-groups, and (*b*) the possibility of a reaction between the *iso*cyanate and groups in insulin other than free amino-groups. It is with the latter problem that we are mainly concerned in this paper.

It is rather difficult to decide, without further investigation, which groups in a protein are likely to react with aryl *iso*cyanates or undergo some modification during the reaction, but evident possibilities are: the hydroxyl group of tyrosine, the disulphide group of cystine, the sulphydryl group of cysteine, the iminazole group of histidine, the guanidine group of arginine, the pyrrolidine group of proline and the acid-amide groups of asparagine and glutamine. Phenyl *iso*cyanate was caused to react with these amino-acids as far as possible under conditions similar to those employed in the preparation of phenylcarbamido-derivatives of proteins. The amino-acid was dissolved in phosphate buffer at *p*H 8 or in 0·2 M $NaHCO_3$, and phenyl *iso*cyanate (1·4 mol. for each group in the amino-acid which might conceivably react with the *iso*cyanate), or the corresponding amount of *p*-bromophenyl *iso*cyanate dissolved in ether, was added to the stirred cooled solution. After 2½–4 hours, this period being longer than that allowed for the preparation of phenylcarbamido-proteins and very considerably longer than that necessary for the inactivation of insulin by phenyl *iso*cyanate, the mixture was centrifuged. (This prolongation was made in order to ensure maximum reaction of the *iso*cyanate with the various amino-acids.) In most cases the phenylcarbamido-acid was precipitated from the supernatant solution by dil. H_2SO_4, but in some instances this method had to be modified (see below).

Certain of these phenylcarbamido-derivatives have been converted into the corresponding hydantoins. These hydantoins were prepared in connexion with investigations on the products obtained by the acid hydrolysis of phenylcarbamido-derivatives of insulin and other proteins, and where the analytical data obtained offer confirmatory evidence for the identification of the phenylcarbamido-derivative, a brief description of the preparation is given.

Tyrosine. Gumpert [1885] showed that the reaction between phenyl *iso*cyanate and phenols proceeds to a slight extent in the cold but more completely on the water-bath. Working at higher temperatures, Goldschmidt & Meissler [1890], Dieckemann *et al.* [1904] and Michael [1905] have obtained similar reactions with phenol, phloroglucinol and hydroresorcinol but from the work of these authors no significant reaction of this type appears to take place at ordinary temperatures.

In our experiments there has been no suggestion of any reaction between phenyl *iso*cyanate and the phenolic group of tyrosine, even when a large excess of the *iso*cyanate is used. Under the usual conditions for the preparation of phenylcarbamido-acids, phenyl and *p*-bromophenyl *iso*cyanates react with tyrosine to give monosubstituted compounds whose N and Br contents agreed with the formulae

$$C_6H_4OH.CH_2.CH.(NH.CO.NHPh).COOH$$

and $$C_6H_4OH.CH_2.CH.(NH.CO.NHC_6H_4Br).COOH$$

[Hopkins & Wormall, 1934, 1].

On account of the low solubility of tyrosine it was not possible to reproduce exactly the same conditions for the preparation of its phenylcarbamido-derivatives as for those of proteins and all other amino-acids used. In the case of tyrosine it is necessary to start with a more alkaline medium, but after the reaction has proceeded for a short time the *p*H is lowered considerably. The yields of the tyrosine derivatives were similar to those obtained in the preparation of the corresponding derivatives of the simple amino-acids (glycine and alanine), and it does not seem probable that any other product is formed in significant amounts.

The phenyl- and *p*-bromophenyl-carbamido-derivatives of tyrosine give well-marked Millon's reactions, and they also give red or black pigments when subjected to the action of tyrosinase, these reactions being indicative of a phenolic grouping [cf. Raper, 1928]. The red pigments produced by the action of the enzyme on the phenyl- and *p*-bromophenyl-carbamido-derivatives are much more stable than is the corresponding pigment from tyrosine, and in this respect these substances appear to behave like tyramine and 3:4-dihydroxyphenylethylmethylamine (epinine), which give more stable red pigments than does tyrosine [Dulière & Raper, 1930].

In all these enzymic experiments, the *p*H of the medium was controlled, the reactions being carried out at *p*H 6, 7 or 8 in the presence of a phosphate buffer.

These experiments appear to offer satisfactory evidence that in the inactivation of insulin by phenyl *iso*cyanate there is no reaction between the latter and the hydroxyl groups of the tyrosine groups in insulin. This is of special interest in view of the recent work of Harington & Neuberger [1936], in which evidence has been obtained that "the phenolic groups of insulin are of importance in relation to its physiological activity". Jensen *et al.* [1936] also discuss the significance of phenolic groups in reference to the hypoglycaemic power of insulin.

Cysteine and cystine. The cystine derivatives have been made previously [Hopkins & Wormall, 1934, 1] and no evidence was obtained of any change in the disulphide linkage.

The preparation of the cysteine derivatives has presented more difficulty on account of the relative insolubility of their sodium salts. Ultimately, however, by the use of a much larger volume of bicarbonate solution, the phenylcarbamido-derivative of cysteine was made in the following manner: 2 ml. (2·8 mol.) of phenyl *iso*cyanate were added to a cooled solution of 1 g. (1 mol.) of cysteine hydrochloride in 100 ml. of 0·2 *M* $NaHCO_3$ with the *p*H adjusted and maintained at about 8. The mixture was stirred for about 2 hours and filtered. The clear filtrate was acidified with 2*N* HCl and the precipitated product immediately recrystallized thrice from dilute alcohol, giving needles or long prisms, M.P. 135–136° (decomp.). The yield of the pure product was 1·6 g. (Found (Roth): N, 10·86; S, 7·92%. Calc. for $C_{17}H_{17}O_4N_3S,C_2H_5OH$: N, 10·37; S, 7·90%.) After being heated at 104° for 4½ hours the product (M.P. 139–140°, decomp.) contained N, 11·90; S, 8·88%. Calc. for $C_{17}H_{17}O_4N_3S$: N, 11·70; S, 8·91%.

The analytical figures indicated that both the amino-group and the SH group of cysteine had been blocked by phenyl *iso*cyanate. The compound gave a very faint nitroprusside reaction due apparently to the action of the NH_3, since other tests have shown that the phenylcarbamidothio-linkage is alkali-labile.

In view of the importance attached by many authors to the sulphur-containing groupings of insulin [cf. Jensen & Evans, 1934] other experiments have been carried out to determine whether these groups are acted upon by phenyl *iso*cyanate. In a typical experiment of this type, phenyl *iso*cyanate (2·8 mol.) was added to cooled solutions of cysteine and of cystine (1 mol.) in $NaHCO_3$ at *p*H 8. The mixtures were shaken for 2 hours and the nitroprusside test for SH groups was carried out at intervals on small samples of the mixtures and on control solutions of cysteine and cystine in $NaHCO_3$. The cystine preparation gave no colour with the nitroprusside at any time: the solution containing cysteine showed rapid diminution of the nitroprusside reaction and after ½ hour nearly all the SH groups had been removed.

This rapid action of phenyl *iso*cyanate on SH groups is interesting, particularly as it takes place under such mild conditions. The authors could find no reference in the literature to such a reaction, although Snape [1885] had prepared the phenyl *iso*cyanate derivative of phenylmercaptan by prolonged heating of a mixture of the two compounds. Further confirmation of this reaction between SH groups and phenyl *iso*cyanate was obtained by a study of the action of the *iso*cyanate on thiolacetic and α-thiolpropionic acids.

α-Thiolpropionic acid (1 mol.) in $NaHCO_3$ solution at *p*H 8 was treated with phenyl *iso*cyanate (1·4 mol.) under the normal conditions. After 1 hour, the mixture was filtered and the clear filtrate acidified with conc. HCl to precipitate a white crystalline solid. (Yield 80 %.) The product was recrystallized three times from dilute alcohol, yielding small needles or prisms, M.P. 140–141° (decomp.). (Found (Roth): N, 6·44; S, 14·43 %. Calc. for $C_{10}H_{11}O_3NS$: N, 6·23; S, 14·22 %.)

The thiolacetic acid compound was prepared in a similar manner in 80 % yield. It was recrystallized from dilute alcohol as long thin prisms, M.P. 144–146° (decomp.). (Found (Roth): N, 6·64; S, 14·84 %. Calc. for $C_9H_9O_3NS$: N, 6·64; S, 15·18 %.)

Proline. The phenylcarbamido-derivative of proline has been described by Fischer [1901]. In the present investigation, the *p*-bromophenylcarbamido-derivative was obtained by a slight modification of the usual technique, the yield of crude product being about 70 % of the theoretical. On recrystallization from dilute alcohol it gave colourless long plates or prisms, M.P. 169° (decomp.). (Found (Roth): Br, 25·41; N, 9·04 %. Calc. for $C_{12}H_{13}O_3N_2Br$: Br, 25·56; N, 8·96 %.)

Histidine. Histidine dihydrochloride (1 mol.) was treated with an ethereal solution of *p*-bromophenyl *iso*cyanate (4·2 mol.) under the usual conditions and the mixture centrifuged after 2½ hours. The precipitate was washed several times with water containing a little Na_2SO_4 and sufficient NaOH to give *p*H 8, and the washings were added to the supernatant solution previously obtained. This solution was evaporated to small bulk *in vacuo*, and treated with conc. and finally 2*N* H_2SO_4 to give maximum precipitation of a white crystalline material. This preparation was dried *in vacuo*. Yield 1·9 g. from 1·2 g. of histidine dihydrochloride. Recrystallized twice from hot water it gave colourless plates, M.P. 177–178° (decomp.). For analysis, the substance was heated at 102–103° for 4 hours. (Found (Roth): N ,15·56; Br, 22·04 %. Calc. for $C_{13}H_{13}O_3N_4Br$: N, 15·87; Br, 22·63 %.)

Arginine. Some difficulty has been experienced in isolating the pure phenyl-

and *p*-bromophenyl-carbamido-derivatives of arginine, and from the results so far obtained it appears probable that when phenyl- and *p*-bromophenyl-*iso*-cyanates act on arginine mixtures of mono- and di- derivatives are produced.

Further work is being carried out to determine to what extent, and how rapidly, phenyl *iso*cyanate reacts with the guanidino-group of arginine and with the same group of arginine-containing proteins. The evidence so far available indicates that in the case of arginine even when a large excess of the *iso*cyanate is used, this reaction is less rapid and less complete than that involving the α-amino-group. In the reaction between the *iso*cyanates and insulin (and other proteins), under the conditions used in this and previous investigations, it is believed that no significant change occurs in the guanidino-grouping, but further work will be necessary before this can be definitely established.

Asparagine. Phenyl *iso*cyanate and asparagine under the usual conditions yielded a phenylcarbamido-derivative. Yield (of crude product) 70% of theoretical. On recrystallization from alcohol it gave prisms, M.P. 163° (decomp.). (Found (Roth): N, 16·50%. Calc. for $C_{11}H_{13}O_4N_3$: N, 16·73%.)

When heated on a water-bath with 5*N* HCl for 6 hours, NH_3 was split off and phenylhydantoinacetic acid was obtained, M.P. 231–233°. (Found (Roth): N, 12·13%. Calc. for $C_{11}H_{10}O_4N_2$: N, 11·96%.)

The *p*-bromophenylcarbamido-derivative, recrystallized twice from alcohol as fine needles, contained 1 mol. of alcohol of crystallization, M.P. 175–176° (decomp.). (Found: Br, 21·18%. Calc. for $C_{11}H_{12}O_4N_3Br,C_2H_6O$: Br, 21·25%.) (After being heated at 102° for 2 hours, this product gave N, 12·68% (Roth). Calc. for $C_{11}H_{12}O_4N_3Br$: N, 12·73%.)

On treatment with hot 5*N* HCl, as above, a good yield of *p*-bromophenylhydantoinacetic acid was obtained. This hydantoin was recrystallized twice from alcohol and gave fine needles with M.P. 220°. (Found after heating at 102–103° for 3 hours (Roth): N, 8·94; Br, 24·91%. Calc. for $C_{11}H_9O_4N_2Br$: N, 8·99; Br, 25·53%.)

In these preparations, as in those of the glutamine derivatives, excess of phenyl- or *p*-bromophenyl-*iso*cyanate was used (2·8 mol. of *iso*cyanate per mol. of asparagine or glutamine). Even with this excess, there appears to be no reaction under the conditions of these experiments between the *iso*cyanate and the acid-amide group.

Glutamine. The phenylcarbamido-derivative of synthetic glutamine [Bergmann *et al.*, 1933] was prepared in the usual manner. The washings from the precipitate and the original supernatant solution were combined and concentrated *in vacuo* at the ordinary temperature. The product was precipitated by H_2SO_4 and the yield of crude substance was about 70%. Two recrystallizations from alcohol gave needles, M.P. 161° (decomp.). (Found (Roth): N, 14·50%. Calc. for $C_{12}H_{15}O_4N_3$: N, 15·85%.)

The low N content of this preparation is most probably due to a loss of amide-N during recrystallization.

The corresponding *p*-bromophenylcarbamido-derivative was obtained as small needles after two recrystallizations from alcohol, M.P. 189°. (Found (Roth): N, 12·08; Br, 22·88%. Calc. for $C_{12}H_{14}O_4N_3Br$: N, 12·21; Br, 23·22%.)

On treatment with hot HCl, the phenylcarbamido-derivative yielded phenylhydantoinpropionic acid. Long prisms from water; M.P. 160–161°. (Found (Roth): N, 11·63%. Calc. for $C_{12}H_{12}O_4N_2$: N, 11·29%.)

The hydantoin obtained from the corresponding bromo-derivative gave long prismatic crystals (from alcohol), M.P. 200–201°. (Found (Roth): N, 8·49; Br, 24·57%. Calc. for $C_{12}H_{11}O_4N_2Br$: N, 8·56; Br, 24·43%.)

DISCUSSION.

The primary object of the earlier part of the work described in the present paper was to find out whether differentiation of hypoglycaemic and hypophosphataemic powers of insulin similar to that described by Davis *et al.* [1933] could be obtained by some other method. For reasons mentioned above, phenyl *iso*cyanate was used as the inactivating agent.

Typical results such as are given in Tables II and III, have failed to demonstrate any significant difference between these two activities of insulin. Thus the inactivation by phenyl *iso*cyanate of the hypoglycaemic activity of insulin, an inactivation which appears to involve principally, if not entirely, the free amino-groups of the insulin [Hopkins & Wormall, 1934, 2; Jensen *et al.* 1934], has in all our experiments been accompanied by a corresponding inactivation of the hypophosphataemic power. This parallelism between the two inactivation processes has been observed when varying amounts of phenyl *iso*cyanate have been used and also when the reaction between the insulin and the *iso*cyanate has been stopped, as far as this is possible, at different stages.

These results do not, of course, offer any strong evidence against the view of Davis *et al.* that insulin owes its multiple activity (i.e. power to reduce the blood glucose, phosphate and amino-acid) to a number of "active groups", "centres" or "units" which vary in stability towards inactivating agents. It appears highly probable that even if there are two or more activities associated with different parts of the insulin molecule, differentiation between these activities will not be possible with every inactivating agent. Numerous failures to demonstrate a difference by other methods will not suffice, therefore, to destroy the value of a single well-established differentiation. It seems highly desirable, however, that confirmation of the results of Davis *et al.* should be obtained, either by the use of the same methods which they used, or preferably by some other method of inactivation. As far as the present authors are aware, there is little or no further evidence that the hypophosphataemic action of insulin is due to anything other than the change which the insulin effects in the carbohydrate metabolism of the body.

The previously mentioned observations of Savino [1924] appear to show that the injection of insulin might cause a fall in the blood phosphate even if the normal blood sugar level is maintained by the injection of glucose; this result however might well be explained as follows. The injected insulin causes an acceleration of tissue oxidative and synthetic changes involving glucose, and this leads to the withdrawal of inorganic phosphate from the blood. Under normal circumstances therefore the lowering of the blood sugar and the reduction in blood phosphate following the injection of insulin should be closely related, but the maintenance of the blood sugar at its normal level, by the injection of glucose, would not prevent the reduction in the phosphate value.

The main conclusion which can be drawn from the experiments described in this paper is that phenyl and *p*-bromophenyl *iso*cyanates destroy the hypophosphataemic power of insulin just as readily as they do the hypoglycaemic activity. Inactivation by these reagents appears to be due to interaction with the free amino-groups of the insulin, and thus these groups appear to be essential components of both hypoglycaemic and hypophosphataemic factors or "centres" of the hormone.

In the second part of this paper, further efforts to determine whether the action of phenyl and *p*-bromophenyl *iso*cyanates on insulin and other proteins is limited to the free amino-groups are described. Experiments have been carried out with various amino-acids to find out whether phenolic groups and basic groups, other than α-amino-groups and the ϵ-amino-groups of lysine, are attacked by these *iso*cyanates. In all these tests, a considerable excess of *iso*cyanate was used, but no evidence was obtained that, under the conditions used for the inactivation of insulin and for the preparation of other phenylcarbamido-protein derivatives, there is any reaction with the following groups; the hydroxyl group of tyrosine, the acid-amide groups of glutamine and asparagine or the iminazole group of histidine. Experiments made with cystine appear to show that no significant change occurs in the S—S group of this amino-acid. In the case of cysteine however rapid condensation with the SH group occurs, but since insulin gives a negative nitroprusside reaction and does not appear to contain free SH groups [cf. Schock *et al.* 1935; Jensen *et al.* 1936], reaction with this group cannot be a contributory cause of the inactivation of the hormone by phenyl *iso*cyanate. The tests made with phenyl *iso*cyanate and arginine show that there may be some interaction with the guanidino-group, but this reaction is probably not as rapid or as complete as the reaction with α-amino-groups or the ϵ-amino-group of lysine. This partial reaction with the guanidino-group of arginine offers special interest in view of the marked hypoglycaemic effect of certain guanidine derivatives, and further work is being carried out in this connexion. From the evidence available at the present time, it does not appear probable that a change in the guanidino-group of insulin is the main or even a contributory cause of the inactivation of insulin by phenyl *iso*cyanate, a process which is very rapid and complete.

Other amino-acids which are known to react with phenyl *iso*cyanate are proline [Fischer, 1901] and hydroxyproline [Fischer, 1902], but since insulin does not contain the latter [Jensen *et al.* 1932], only the former amino-acid need be considered here. Jensen & Evans [1935] obtained evidence of the presence of proline in insulin, but were unable to isolate the phenylhydantoin of this amino-acid from small amounts of hydrolysed phenylcarbamido-insulin. In view of this, and since zein, which contains 9·0 % of proline [Osborne & Liddle, 1910] does not react with *p*-bromophenyl *iso*cyanate [Hopkins & Wormall, 1933, 1] it seems justifiable to conclude that the formation of phenylcarbamido-protein derivatives does not involve any significant change in the proline grouping.

From these results with amino-acids it does not necessarily follow that similar reactions will occur with natural or derived proteins. One is probably justified however in concluding that if phenyl *iso*cyanate does not react with a given group in an amino-acid, there is a strong probability that no reaction will occur with this group when the amino-acid is linked to many others. It is the intention of the authors to carry out a few tests with certain peptides, but there appears to be no strong reason to suspect that the results will differ from those obtained with the simple amino-acids.

In all these later investigations therefore no evidence has been obtained which necessitates modification of the view previously expressed that phenyl *iso*cyanate reacts only with the ϵ-amino-groups of lysine and any free α-amino-groups present in proteins. Furthermore, the close relationship between the bromine contents of *p*-bromophenylcarbamido-derivatives of insulin and other proteins and the theoretical values, calculated from the decrease in free amino-N as a result of the action of the *iso*cyanate or from the free amino-N content of insulin [Hopkins & Wormall, 1933, 1; 1934, 2], appears to be strong evidence against the view that the reaction involves other groupings of the protein. With the majority of native proteins the lysine probably accounts for the whole of the free amino-N, but this is not true for insulin. Jensen & Evans [1935] have produced satisfactory evidence that a further portion, but not the whole, of this additional free amino-N of insulin is present in phenylalanine groupings. Further work is being carried out on the phenylcarbamido-derivatives of insulin and other proteins in the hope that more information will be obtained about these important free amino-groups.

SUMMARY.

1. The hypophosphataemic activity of insulin is destroyed by phenyl *iso*cyanate in the same way as is the hypoglycaemic power.

2. In these experiments, the destruction of the hypoglycaemic power has been parallel with the destruction of the hypophosphataemic power. This relationship has been found (*a*) when varying amounts of phenyl *iso*cyanate were used and (*b*) when the inactivation process was stopped at different stages.

3. Davis *et al.* [1933], using acid alcohol and soft X-rays to inactivate insulin, concluded that different "active groups" of the hormone are responsible for the two activities mentioned above. In the work described in this paper, no similar differentiation has been found possible with phenyl *iso*cyanate as the inactivating agent.

4. Further investigations have been carried out to determine whether the action of phenyl and *p*-bromophenyl *iso*cyanates on insulin and other proteins is confined to the free α-amino-groups. It has been found that these *iso*cyanates do not react, under conditions similar to those maintained in the preparation of phenylcarbamido-protein derivatives, with the hydroxyl group of tyrosine, the acid-amide groups of asparagine and glutamine or the iminazole group of histidine, nor is there any significant change in the S—S linkage. The SH group of cysteine (and similar compounds) reacts with phenyl *iso*cyanate, but it is not necessary to consider this interaction in the case of insulin which contains no free SH groups.

Phenyl *iso*cyanate reacts with the pyrrolidine group of proline and to some extent with the guanidino-group of arginine. From the evidence available, however, it is believed that these reactions, even if they occur when insulin is inactivated by phenyl and *p*-bromophenyl *iso*cyanates, do not account for the inactivation of the hormone.

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CCLXX. STUDIES ON HEPARIN.

IV. OBSERVATIONS ON THE CHEMISTRY OF HEPARIN.

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Since Howell & Holt [1918] demonstrated that dog liver contained a substance, heparin, that retards the coagulation of blood *in vitro*, much work has been done to devise methods for isolating very active preparations of this material. Chemical analyses of such products, however, have differed greatly, owing to the fact that the substance has not yet been isolated in pure form. Howell [1928] obtained a product which was N- and P-free and concluded that heparin was a derivative of glycuronic acid. Schmitz & Fischer [1933] believed heparin to be a trisaccharide $C_{18}H_{32}O_{17}$ containing one carboxylic group. The product isolated by Scott & Charles [1933] gave a positive α-naphthol reaction but the test for glycuronic acids with naphthoresorcinol was negative. Their preparation contained approximately 2 % N and was readily inactivated by nitrous acid. These facts suggested the presence of an amino-group essential for the physiological action of heparin. The more recent work of Jorpes [1935] has contributed much to the chemistry of heparin. His results led him to advance the theory that heparin is a chondroitin polysulphuric acid which would explain the difficulties encountered in attempting to remove inorganic impurities from heparin preparations. He also found that his product contained approximately 2 % N and gave no test for glycuronic acid. Schmitz [1935] more recently has isolated a very active preparation which was not a sulphuric acid derivative and which contained only 4–5 % ash.

Because of the divergence of opinion as to the chemical nature of heparin, much work has been done in these laboratories in an attempt to isolate this substance in pure form. The active substance is completely soluble in formamide and in butylamine but addition of other organic liquids to solutions of heparin in these solvents produces only amorphous precipitates. It was thought that perhaps the failure to crystallize heparin was due to its high ash content. The heparin was therefore prepared as the calcium salt and, after removal of calcium, reprecipitated with glacial acetic acid, yielding a product usually with about 5 % ash. Further attempts to reduce the ash content were unsuccessful until it was found that precipitation of heparin from aqueous solutions by means of benzidine reduced the ash content to 0·7 %. Attempts to crystallize the resultant preparation by means of various solvents or as an alkaloidal salt only resulted in amorphous products. Finally barium acetate was used and a crystalline barium salt was obtained. The details of the procedure used for isolating the crystals as well as their analyses and other chemical findings are recorded below.

Experimental.

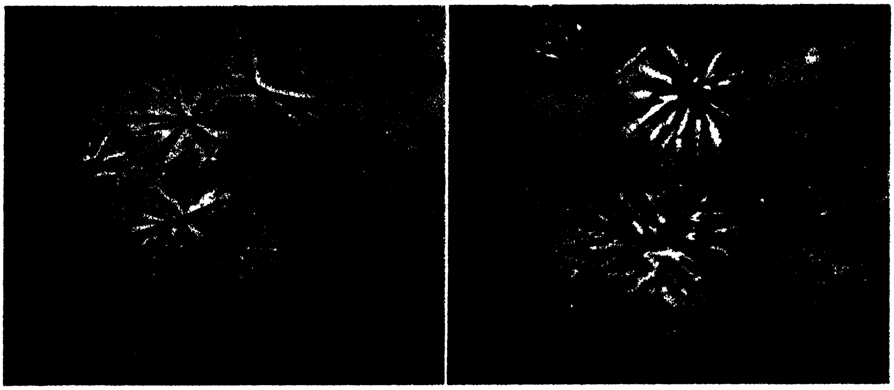
To prepare heparin containing about 60 units per mg. we have used as our initial material crude heparin obtained from ox lung according to the method previously described [Charles & Scott, 1934]. Following our usual procedure

[Scott & Charles, 1933] the crude anticoagulant was then purified by Lloyd's reagent and acetic acid. The product contained 15 units per mg. Using this material highly active preparations of uniform activity were obtained in the following manner.

20 g. of heparin (15 units per mg.) are dissolved in 2 l. of water. The reaction is adjusted to pH 5·0 with glacial acetic acid and 20 g. of Lloyd's reagent are added; after 12–15 hours, the mixture is filtered by suction. The reaction of the filtrate is adjusted with acetic acid to pH 4·0 and 20 g. of Lloyd's reagent are added. After filtration, NaCl is added to 0·85 % followed by acetone (approx. 25 % final concentration) to produce a flocculent precipitate. The precipitate is removed by centrifuging and discarded and the heparin precipitated from the clear centrifugate by the addition of acetone to a final concentration of 66 %. After 12–15 hours, the clear supernatant liquid is removed by filtration and the precipitate washed thoroughly with alcohol and dried. 6 g. of this material are then dissolved in 300 ml. of water and 10 % $CdCl_2$ is added until no further precipitation occurs; after 4–5 hours the mixture is centrifuged and the precipitate rejected. NaCl is then added to the clear liquid to 0·85 % and the heparin precipitated by adding 2 vol. of acetone. The purified heparin is separated by filtration, washed with alcohol and dried. The activity is 60–65 units per mg.

About 70 % of the original potency is recovered in the highly purified form by this method. The preparations, however, contain a larger proportion of inorganic material, the greater part of which can be removed in the following manner. 1 g. of the purified heparin (60 units per mg.) is dissolved in 30 ml. of water and acidified to about pH 5 with acetic acid. To this is added 1 ml. of a saturated solution of ammonium oxalate. The mixture is warmed to 50° and centrifuged. The process of adding oxalate solution, heating and centrifuging is repeated until the addition of ammonium oxalate to the supernatant liquid no longer gives a precipitate. The clear brown supernatant liquid is decanted and evaporated to dryness *in vacuo*. The residue is dissolved in 5 ml. of water and the heparin precipitated by the addition of 45 ml. of acetic acid. After centrifuging the precipitate is redissolved in 5 ml. of water and reprecipitated with 45 ml. of glacial acetic acid. The precipitate is washed with alcohol and ether and then dried, yielding a white powder containing 70–80 units per mg. and about 5 % ash.

Many attempts were made to reduce further the ash of this purified heparin. It seemed likely that the inorganic material was held in combination with the acid groups of heparin. Neuberg & Schuchardt [1935] have shown that benzidine combines with the phosphoric acid group of phosphoglyceric acid and it seemed probable that benzidine might combine with the acid groups of heparin. Furthermore, the benzidine could subsequently be removed from such a complex by treatment with aqueous solutions of sodium or ammonium hydroxide. Using this process, it has been found that the ash content of heparin can be reduced to 0·7 % by the following treatment. 100 mg. of heparin, after the ammonium oxalate treatment, are dissolved in 2 ml. of water and 5 % benzidine hydrochloride in dilute HCl is added, giving a brown tarry precipitate. The addition of the benzidine solution is continued until, after centrifuging, no further precipitate is obtained. The precipitate is washed thoroughly with methyl alcohol and dried. This material is then suspended in water (5 ml. per 100 mg.) and made alkaline with sodium or ammonium hydroxide. The mixture is heated to 75° until a clear brown solution is obtained. When the solution is cooled slowly to 6° the benzidine precipitates and is removed by centrifuging. The supernatant



A photomicrograph of the crystalline barium salt of heparin.
Magnification × 1000.

liquid is brown and contains a few small crystals of benzidine which are dissolved by adding 2 vol. of methyl alcohol. From this solution the heparin is precipitated by 2 vol. of ether and after washing with alcohol and ether is dried. This material is then dissolved in 5 ml. of water and the heparin precipitated by the addition of 45 ml. of glacial acetic acid. The precipitate is washed twice with 90 % acetic acid, then with alcohol and ether. The dried material contains only 0·7 % ash.

Barium salt of heparin. 50 mg. of the heparin of low ash content (0·7 %) are dissolved in 4 ml. of water and 1 ml. of 5 % barium acetate is added. This causes a slight turbidity. After 12–15 hours at 20° a brown tarry inactive material separates. The clear centrifuged supernatant liquid is decanted and to it 1 ml. of glacial acetic acid is added. A white precipitate forms which is dissolved by heating to 70–75°. When cooled slowly to room temperature a crystalline precipitate forms. A photomicrograph of the crystals is shown in Plate IV. The mixture is cooled to 6° for several hours and then centrifuged. The crystals are washed twice with 90 % acetic acid, methyl alcohol and finally with ether; the final traces of ether are removed *in vacuo*. Two samples of crystals were prepared, each obtained from different lots of lung. The results of the chemical analyses of both crystalline preparations are shown in Table I. Samples of the crystals were ashed in the presence of oxygen. To other samples, one or two drops of H_2SO_4 were added before ashing. Since the weights of ash determined by each procedure were practically identical, the inorganic material must have been present as a sulphate. In the preparation of sample I the benzidine was removed by treatment with ammonia, whereas in sample II NaOH was used. The ash contents however, were essentially the same. Since the use of NaOH did not increase the ash content, the inorganic substance forming the ash must be $BaSO_4$. The total S was found by first estimating the S present as volatile SO_3 by the method of Pregl [1930] which was found to be 4·78 %. The residue was then ashed and from the inorganic material ($BaSO_4$) it was found that the non-volatile S was 4·58 %. Hence the total S in the crystals is 9·36 %. These S values indicate that Ba is present in the crystalline salt in the form $RSO_3—Ba—SO_3R$. From such a substance, one-half of the S content would be determinable as an oxide of S, whilst the other half would be estimated as non-combustible material in the form of $BaSO_4$.

Table I. *Analysis of heparin.*

	C %	H %	N %	S %	Ash ($BaSO_4$) %	Ba %
Ba salt of heparin						
Sample I	17·87	3·89	1·63	9·24	33·8	19·9
Sample II	18·00	3·88	1·73	9·36	33·3	19·6
Ba-free heparin (calc.)						
Sample I	22·22	4·84	2·01	11·49	—	—
Sample II	22·39	4·82	2·14	11·64	—	—

The amino-N content of the crystals (Van Slyke) was found to be 0·40 %.

On heating in a platinum boat at 110° in a current of dry air the crystals attained constant weight in 2 hours, the loss in weight being 5 %.

An attempt was next made to remove the Ba and analyse the ash-free material. Again the use of benzidine was found to be the most satisfactory means of accomplishing this.

Benzidine salt of heparin. 200 mg. of crystals are dissolved in 10 ml. of water. Benzidine hydrochloride in dilute HCl is added until no further precipitate forms. This precipitate is washed, first with methyl alcohol, then with ether and dried. The material is suspended in 10 ml. of water and N NaOH added until alkaline. The suspension is heated to 70–75° and the resulting clear solution cooled slowly to 0°. The benzidine is removed by centrifuging, and the heparin precipitated by the addition of 9 vol. of acetic acid. After washing thoroughly with methyl alcohol and ether, the material is dried, redissolved, precipitated again as the benzidine compound and the benzidine removed as described above. The resultant product is dissolved in 7 ml. of water and the benzidine compound again formed. The precipitate is washed thoroughly with alcohol and ether and dried. Analyses of this compound are shown in Table II. Since the S content of Ba-free heparin is 11·56 %, and the benzidine-heparin compound contains 8·46 % S, it can readily be calculated that heparin constitutes 73·3 % of the benzidine-heparin compound. From this relationship the composition of benzidine-free heparin is estimated and is also shown in Table II.

Table II. *Analysis of heparin.*

Material	C %	H %	N %	S %	Ash %
Benzidine-heparin compound	38·05	5·40	5·60	8·46	1·09
Benzidine-free heparin (calc.)	23·09	4·99	2·10	11·54	—

Miscellaneous experiments.

Action of nitrous acid on heparin. The crystalline material when treated with $NaNO_2$ in the presence of acetic acid (pH 4·0) was almost completely inactivated, whilst no inactivation occurred in solutions having pH 7 or higher. Solutions of heparin at pH 4 without $NaNO_2$ showed no loss of potency under similar conditions.

Action of formaldehyde on heparin. The heparin crystals, on treatment with about 100 parts of formalin for 1 hour at 45° at pH 8 and pH 4 lost about 50 % of their activity. Controls run under the above conditions in the absence of formaldehyde showed no loss.

Colour tests. The Molisch test for carbohydrate compounds was positive. The naphthoresorcinol test for glycuronic acid was negative as was Tollens's phloroglucinol reaction for pentoses or glycuronic acid. Control tests showed that heparin did not interfere with these reactions.

Action of acid alcohol on heparin. The benzidine was removed from the benzidine-heparin compound by treatment with ammonia as described above. 10 mg. of this material were dissolved in 1 ml. of 0·1 N HCl in 95 % methyl alcohol. This solution was kept at 10° for 20 hours. The heparin was precipitated by the addition of 9 ml. of glacial acetic acid, washed with ether and dried. A weighed sample of this material was assayed and found to have lost approximately 80 % of its potency. The acetic acid supernatant liquid from the inactivated heparin was evaporated to dryness and the residue redissolved in 1 ml. of water. When tested with barium acetate this solution gave a precipitate indicating the presence of $SO_4^{=}$. A control experiment in which the heparin was precipitated immediately from acid alcohol without loss of potency did not show the presence of $SO_4^{=}$ in the filtrate. When acid water was used instead of methyl alcohol no inactivation of the heparin occurred over 20 hours.

Physiological assay.

In the present investigation the two crystalline samples of heparin were compared physiologically with two standardized preparations. The first standard was prepared in these laboratories and was three times as active as the commercial product of Hynson, Westcott and Dunning. This ratio has been checked many times. The second was the commercial material of Hynson, Westcott and Dunning. This was used so that the unitage of the crystalline heparin could be directly correlated with the more active preparation of Jorpes.

The method of assay was as follows. 3·520 mg. of sample I were dissolved in 3·50 ml. of isotonic saline, and 5·282 mg. of sample II in 5·30 ml. of isotonic saline. Each dilution was diluted further 1:40, 1:45 and 1:50. In order to compare the potency of our material with that of Jorpes, a solution of commercial heparin (Hynson, Westcott and Dunning) was made containing 3·643 mg. in 7·30 ml. This latter solution was used without further dilution. Into a series of tubes 0·1, 0·2 and 0·3 ml. of each solution was measured. Isotonic saline was added where necessary to bring the volume in each tube to 0·3 ml. By means of a cannula inserted in the carotid artery of a cat anaesthetized with sodium amytal, 0·7 ml. of blood was added to each tube. The tubes were kept at 20° for 2 hours and then examined. It was found that sample I or sample II in a concentration of 1 mg. in 45 ml. showed the same potency as the control solution containing 1 mg. in 2·0 ml. Hence the ratio of the activity of the crystals to that of the commercial product of Hynson, Westcott and Dunning is approximately 22:1. Thus the crystals are about twice as active as the purest preparation isolated by Jorpes. When the assay was performed by the method formerly described by us [Scott & Charles, 1933] the crystals had a potency of approximately 500 units per mg.

Discussion.

A method of obtaining very active heparin preparations has been described. Using this procedure a quantity of heparin was purified from different lots of ox lung. After reducing the ash content of these preparations to 0·7 % by means of ammonium oxalate and by benzidine, the crystalline barium salt was formed. Analyses of each lot of crystals are in agreement (Table I) and when calculated on a Ba-free basis, the results indicate that the empirical formula of heparin can be expressed as $C_{25}H_{65}O_{50}N_2S_5$. From the analytical and qualitative results it appears that S is present in heparin in the form $—SO_3H$. The positive Molisch reaction indicates that a carbohydrate grouping is present in crystalline heparin. Tests with naphthoresorcinol and with phloroglucinol were negative, indicating the absence of glycuronic acid and pentoses. The fact that negative tests were obtained substantiates our former findings and agrees with the more recent results of Jorpes. The possibility that the carbohydrate is combined with the $—SO_3H$ groups as in chondroitin sulphuric acid has been suggested by Jorpes. Indirect evidence that such may be the case is indicated by the work of Bergstrom [1936] who showed that certain polysaccharides, when sulphonated, had a definite anticoagulant action on blood. Further, our work shows that when ash-free heparin was treated with methyl alcohol containing a small amount of HCl, (0·1*N*), at 10° the potency was destroyed and that this inactivation was accompanied by the liberation of $SO_4^{=}$. Gebauer-Fuelnegg & Dingler [1930] have shown that $—SO_3H$ groups can readily be removed from sulphonated cellulose by treatment with acid methyl alcohol. The fact that the potency of the heparin inactivated by methyl alcohol could not be recovered with aqueous or alcoholic NaOH indicates that the loss in potency was not due to esterification. Finally, it should be noted that the analyses are not in agreement with Jorpes' view that heparin is a chondroitin polysulphuric acid, the carbon content of heparin being much too low. However, it is quite possible that heparin is very similar in structure to chondroitin sulphuric acid.

From the crystalline salt of heparin an amorphous benzidine-heparin compound was prepared. The composition of heparin calculated from the analysis of this compound (Table II) is practically identical with that estimated for ash-free heparin from the Ba salt (Table I).

The crystalline Ba salt of heparin contains N, part of which is associated with the anticoagulant action, as shown in experiments with nitrous acid or formaldehyde. This confirms our earlier findings [Scott & Charles, 1933]. The inactivation does not appear to be due to acidity since the control solutions showed no loss in potency. Neither can the inactivation be ascribed to oxidation or reduction since heparin is comparatively stable to such reagents. An estimation of amino-N (Van Slyke) showed that only about 25 % of the N was present in this form.

The physiological activities of heparin preparations obtained by different workers are difficult to correlate. This is largely due to the fact that different methods of assay have been used. In these laboratories known amounts of heparin were mixed with cat blood and the samples kept for a definite time before being examined. Jorpes, on the other hand, used ox blood and inverted the tubes at certain intervals during the test. Fischer & Schmitz [1935] have defined the unit in terms of the clotting time of a system of hen plasma and muscle extract. These considerations, together with the fact that no common standard preparation has been used, have contributed to a lack of uniformity in expressing the unitage of heparin preparations. It seems logical that the unit should be expressed in terms of a highly active material which can readily be prepared and which is of uniform composition and activity. Further, definite conditions for the assay of heparin should be established with the object of obtaining the most reliable method for the comparison of different preparations. The crystals, when assayed physiologically by the method formerly described [Scott & Charles, 1933], showed a potency of about 500 units per mg. In order to obtain a relation between the potency of Jorpes's most active preparation and the crystalline material, the activity of the latter was compared with the commercial product of Hynson, Westcott and Dunning. It was found that the crystals were 22 times as active as the commercial product. This is about twice the potency of Jorpes's preparation.

SUMMARY.

A method has been described for obtaining very active preparations of heparin. Benzidine was found to be most satisfactory for removing inorganic materials. The crystalline barium salt was formed and analyses showed that the empirical formula of heparin could be expressed as $C_{25}H_{65}O_{50}N_2S_5$. An amorphous benzidine-heparin compound was formed, analysis of which gave the same empirical formula for heparin. Evidence has also been presented confirming the finding of Jorpes that the sulphur is present in heparin as $—SO_3H$ groups. The crystalline product contained nitrogen, part of which was present as $—NH_2$ groups. Experiments with nitrous acid and formaldehyde indicated that the amino-nitrogen was associated with the physiological activity of heparin. Certain colour tests indicated the presence of a carbohydrate complex and the absence of pentoses and glycuronic acid. Potency assays showed that the crystalline preparation was 22 times as active as the commercial standard used by Jorpes.

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CCLXXI. STUDIES IN THE METABOLISM OF THE STRICT ANAEROBES (GENUS *CLOSTRIDIUM*).

V. FURTHER EXPERIMENTS ON THE COUPLED REACTIONS BETWEEN PAIRS OF AMINO-ACIDS INDUCED BY *CL. SPOROGENES*.

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In the first paper of this series Stickland [1934] showed that washed cells of *Cl. sporogenes* were able to bring about the linked oxidation and reduction of pairs of amino-acids. He considered that these reactions were probably the source of energy for the growth of this organism on protein digest media. The amino-acids that were activated as H donators, i.e. oxidized, were: *d*-alanine, *l*-leucine, *d*-valine, and to a less extent *l*-histidine, *l*-phenylalanine and some others. The amino-acids activated as H acceptors, i.e. reduced, were: glycine, *l*-proline and *l*-hydroxyproline. Reactions occurred between any H donator and any H acceptor in the presence of washed cells of *Cl. sporogenes*. Serine, tyrosine and to a small extent, glycine were broken down in the absence of any other amino-acid. In following papers Stickland [1935, 1, 2, 3] worked out in detail the chemistry of some of these linked reactions.

The work of Fildes & Richardson [1935] makes it almost certain that the "Stickland reaction" is in fact the main source of energy for the growth of these organisms. These workers were able to obtain good growth of *Cl. sporogenes* on a medium consisting of pure amino-acids, salts and "*sporogenes* vitamin" only. Furthermore they found that "the conclusions of Stickland upon the sources of energy available for this organism are in agreement with growth experiments".

Bessey & King [1934] have found that arginine, alanine, glutamic acid, tyrosine and cystine are deaminated when incubated alone with washed suspensions of *Cl. sporogenes*. In the cases of alanine and glutamic acid the apparent discrepancy between their results and those of Stickland is possibly explained by the duration of the experiments (3–10 days) and the small amount of substrate used ($M/300$). It seems possible that in Bessey & King's experiments alanine and glutamic acid, which are H donators, were deaminated by reacting with H acceptors formed by the autolysis of the bacterial suspension itself. The case of arginine and cystine will be discussed later.

The work reported in the present paper was undertaken in an attempt to correlate chemical structure with the power of acting as H donator or as H acceptor in the "Stickland reaction". In the course of the experiments it was found that ornithine and arginine acted powerfully as H acceptors and in addition were partially deaminated by *Cl. sporogenes* in the absence of other amino-acids. Similarly it was found that cysteine was deaminated alone in addition to being activated as H donator. The ornithine reaction will be dealt with fully in the present communication; preliminary experiments with cysteine and arginine are also reported.

Preparation of bacterial suspensions.

The organism used was *Cl. sporogenes* (Bellette) and was obtained from the National Collection of Type Cultures (No. 533). This is the same strain as that used by Stickland [1934] and by Fildes & Richardson [1935]. If the suspensions were prepared as described by Stickland [1934] they were found to be occasionally inactive (10 % of cases). The following procedure gave better growth and more consistently active suspensions. 900 ml. tryptic caseinogen digest broth were enriched by the addition of 2 g. wet weight of Lab-lemco (beef extract) and sown immediately after autoclaving and rapid cooling from a culture of *Cl. sporogenes* on Robertson's meat medium. The flask was then put at once into a McIntosh & Fildes's anaerobic jar and incubated at 38°. Under these conditions good growth and active suspensions were obtained after only 20 hours; longer incubation led to spore formation and less active suspensions. The 20-hour cultures were therefore spun down on the centrifuge and the bacteria washed twice on the centrifuge with Ringer's solution. The cells from 900 ml. broth were usually finally suspended in 20 ml. Ringer's solution.

Experimental methods.

In order to determine whether a given substance could be activated by the organism either as H donator or acceptor, use was made of the fact that during these coupled reactions the amino-acids are often deaminated with liberation of ammonia. In testing a substance for activity as H donator it was incubated in the presence of *Cl. sporogenes* with an H acceptor known to give rise to ammonia on reduction (e.g. glycine). Similarly in testing for an H acceptor the substance was mixed with an H donator known to give rise to ammonia on oxidation (e.g. alanine). A control was always done with two amino-acids known to react, such as alanine and proline, in order to be certain that the bacterial suspension was active.

Ammonia was estimated as described by Stickland [1934] by distilling 2 ml. samples from 50% alcohol-borate buffer *p*H 10 mixture into $N/100$ H_2SO_4 and back-titrating the latter with $N/100$ CO_2-free NaOH. All experiments were done anaerobically at 37° in evacuated Thunberg tubes.

EXPERIMENTAL RESULTS.

l-*Cysteine.*

Stickland [1934] found that serine acted as H donator to glycine in addition to being partially deaminated when incubated alone with *Cl. sporogenes.* In view of the close structural relationship of serine and cysteine it was decided to test the action of the organism on the latter amino-acid. Bessey & King [1934] found that cystine was deaminated to the extent of 15 % by washed cells of *Cl. sporogenes* and Tarr [1933] has reported a 43 % formation of H_2S. The cysteine solution used in the following experiments was prepared from *l*-cysteine hydrochloride and was neutralized just before use in order to avoid autoxidation. Thunberg tubes were set up containing 0·5 ml. $M/5$ phosphate buffer *p*H 7, 1 ml. bacterial suspension, substrates as specified below and water to a total volume of 2·5 ml. Control tubes containing no substrate were also put up. Sets of tubes were taken out of the bath at intervals and the ammonia formed estimated. After 3 hours there was no increase in ammonia in any case and the results obtained are summarized in Table I. In each case the no-substrate control has been deducted (a small amount of NH_3 is always formed from the autolysis of the

Table I.

Substrate	NH_3 formed as ml. $N/100$
(1) *l*-Cysteine	2·57
(2) *d*-Alanine	0
(3) *l*-Proline	0
(4) Glycine	0·09
(5) *d*-Alanine + *l*-proline	4·78
(6) *d*-Alanine + *l*-cysteine	2·59
(7) *l*-Cysteine + *l*-proline	3·43
(8) *l*-Cysteine + glycine	3·72
(9) *d*-Alanine + *l*-proline minus (2) and (3)	4·78
(10) *d*-Alanine + *l*-cysteine minus (1) and (2)	0·02
(11) *l*-Cysteine + *l*-proline minus (1) and (3)	0·86
(12) *l*-Cysteine + glycine minus (1) and (4)	1·06

Quantities used: cysteine and alanine, 0·5 ml. $M/10$; glycine and proline, 0·5 ml. $M/5$.

suspensions). Qualitative tests for H_2S were positive in all tubes containing cysteine. The results show that cysteine is only 50 % deaminated (1), since 0·5 ml. $M/10$ cysteine would yield 5 ml. $N/100$ NH_3 on complete deamination. The proline-alanine control (9) was complete in 3 hours and longer incubation of the cysteine tubes (10 hours) gave no increase in NH_3. This fits in with the approx. 50 % formation of H_2S found by Tarr [1933]. In addition to the deamination which occurs when cysteine is incubated alone with the bacterium, some extra ammonia beyond the controls is formed when cystine is mixed with glycine or proline, (11) and (12); cysteine therefore also acts as H donator. No extra ammonia was produced from cysteine-alanine mixtures (10) showing that cysteine does not act as H acceptor. The behaviour of cysteine was entirely analogous to that found for serine by Stickland [1934]. The metabolism of cysteine (and cystine) by *Cl. sporogenes* is being further investigated.

Glycollic acid.

Glycine has been shown by Stickland [1934] to act as a H acceptor. The question arises whether the substitution of a OH group for the NH_2 group of glycine destroys this power. The experimental details were the same as for the cysteine experiments. The results showed that glycollic acid can act neither as donator to glycine nor as acceptor to alanine. The alanine-proline control showed that the bacterial suspension was active. It is evident that the replacement of the NH_2 of glycine by OH completely destroys its capacity as H acceptor in the presence of *Cl. sporogenes*.

β-Alanine.

In order to determine the effect on the activity of alanine as H donator of shifting the NH_2 group from the α- to the β-position, experiments were carried out with β-alanine. The general plan of the experiments was again the same as with cysteine. The results showed (*a*) that β-alanine was not deaminated when incubated alone with the organism, and (*b*) that no NH_3 was formed from mixtures of β-alanine and proline or of α-alanine and β-alanine, indicating that β-alanine acts neither as H donator nor as H acceptor in the "Stickland reaction".

Similar experiments with taurine, $CH_2NH_2CH_2SO_3H$, showed this acid also to be inactive as either donator or acceptor.

d-Ornithine.

l-Proline [Stickland, 1934] acts as H acceptor and gives rise to δ-aminovaleric acid on reduction [1935, 1]. Ornithine has a similar C chain to proline and there is some evidence [Ackermann, 1907–10] that it may also give rise to δ-aminovaleric acid on putrefaction by mixed cultures of bacteria. The action of washed suspensions of *Cl. sporogenes* on ornithine was therefore tested. The ornithine solutions used throughout this section were prepared from *d*-ornithine hydrochloride (Hoffmann La Roche) and were carefully neutralized before use.

Preliminary experiments. (*a*) *Activity as H acceptor.* Thunberg tubes were set up containing 1 ml. phosphate buffer *p*H 7, 1 ml. bacterial suspension, substrates as specified below and water to a total volume of 4 ml. Controls containing (*a*) no substrate and (*b*) no bacterial suspension were also put up. After subtracting the no-substrate blank the following results were obtained after 3·5 hours' incubation:

Substrate	NH_3 formed as ml. $N/100$
(1) 1 ml. $M/10$ *d*-alanine	0·2
(2) 1 ml. $M/10$ *d*-ornithine	7·2
(3) 1 ml. $M/10$ *d*-alanine + 1 ml. $M/10$ *d*-ornithine	15·7
(4) Alanine + ornithine minus (1) and (2)	8·3
(5) Alanine + ornithine (no bacterial suspension)	0

It will be seen (2) that ornithine is deaminated to a considerable extent when incubated alone with the organism, but in addition it forms a large amount of extra NH_3, beyond the controls, when mixed with alanine (4). Ornithine therefore acts as H acceptor to alanine. There is no NH_3 formation in the absence of bacterial suspension (5).

The fact that ornithine is activated by *Cl. sporogenes* as H acceptor was confirmed by the indicator method. It can be shown that ornithine is able to reoxidize (i.e. accept H from) reduced benzylviologen in the presence of the bacterial cells. Hollow-stoppered Thunberg tubes were set up containing in the main tube 1 ml. buffer *p*H 7, 1 ml. $M/2000$ benzylviologen and 1 ml. bacterial suspension. The stoppers contained 0·5 ml. $M/5$ substrate. The tubes were thoroughly evacuated and incubated until the dye had been completely reduced by the reducing blank of the bacterial suspension. The contents of the stoppers were then tipped in and the time taken for complete reoxidation of the dye noted. The results obtained were:

Substrate	Reoxidation time
Water	Not reoxidized
l-Proline	3 min.
d-Ornithine	3 min.

(*b*) *Activity as H donator.* Ornithine was also tested for activity as H donator by incubation with the acceptors glycine and proline. The considerable deamination of ornithine when incubated alone with the organism was confirmed, but no extra ammonia was formed in the presence of either glycine or proline indicating that ornithine has no H-donating action. This result was confirmed by methylene blue reduction experiments.

Oxidizing level of ornithine. Alanine, in the presence of *Cl. sporogenes*, is able to reduce oxidation-reduction dyes down to benzylviologen (*r*H 3 at *p*H 7·5), while it was shown above that ornithine is able to reoxidize reduced benzylviologen. An oxido-reduction reaction between these two amino-acids is therefore possible and, as has been shown in the preliminary experiments, does in fact take place. The exact oxidizing level of the ornithine system was estimated by indicator reoxidation experiments similar to those described above with benzylviologen; if the reducing blank of the suspension was insufficient to reduce the indicator 0·5 ml. $M/200$ alanine was added. Controls in which water was tipped in instead of ornithine were done in each case. The following results were obtained:

Indicator	rH of indicator	Reoxidation
Benzylviologen	3 (at pH 7·5)	+ +
Neutral red	3·0	+ +
Rosinduline	4·5	+ +
Phenosafranine	5·5	+ +
Ethyl Capri blue	11·5	—

+ + indicates complete reoxidation.

Ornithine is therefore able to reoxidize all leuco-dyes up to phenosafranine (rH 5·5) but fails to reoxidize leuco-ethyl Capri blue (rH 11·5) so that the rH of the ornithine system lies between these values; no suitable indicator for use with *Cl. sporogenes* between these values exists [Stickland, 1934]. Comparison of these results with those given by proline [Stickland, 1934] shows that the potential levels of these two systems, as far as dye experiments can show, must be fairly close.

Reaction of ornithine with various H donators. Proof has already been given that ornithine will react with alanine. The next series of experiments was designed to test whether ornithine also reacts with the other chief H donators found by Stickland. Thunberg tubes were set up containing 0·5 ml. buffer pH 7, 1 ml. bacterial suspension, 0·5 ml. $M/10$ H donator, 0·5 ml. $M/10$ ornithine and water to 2·5 ml. Controls (*a*) with no substrates, (*b*) with each donator alone, and (*c*) with ornithine alone were also carried out. The results obtained after 5 hours' incubation are summarized in Table II, the appropriate control values having been deducted in each case. In the third column the relative efficiencies of H donators are compared by setting leucine as 100. The results show general correspondence with those obtained by Stickland [1934] in a similar study using glycine as acceptor.

Table II.

H donator	NH_3 formed, ml. $N/100$	Leucine = 100	Glycine as acceptor (Stickland)
d-Alanine	2·50	95	100
l-Leucine	2·64	100	70
d-Valine	1·93	73	100
l-Histidine	1·00	38	55
l-Phenylalanine	0·28	11	30

The product of the reduction of ornithine.

Proline, according to Stickland [1935, 1], gives rise to δ-aminovaleric acid on reduction; ornithine has a similar C chain and there is some evidence from old work of Ackermann [1907–10] that it may give rise to δ-aminovaleric acid by the action of mixed putrefactive organisms. It has already been established (p. 1937) that some deamination occurs when ornithine is reduced. As a working hypothesis it may be postulated that the α-amino-group of ornithine is deaminated during reduction and that δ-aminovaleric acid is the product. In such a reaction 2 atoms of H could be accepted:

$$CH_2NH_2.CH_2.CH_2.CHNH_2.COOH + 2H = CH_2NH_2.CH_2.CH_2.CH_2.COOH + NH_3.$$

It is known [Stickland, 1935, 2] that alanine is oxidized in the presence of acceptors according to the following equation:

$$CH_3.CHNH_2.COOH + 2H_2O = CH_3.COOH + NH_3 + CO_2 + 4H.$$

Since 4H are donated by alanine, 2 mol. of ornithine would be required to oxidize 1 mol. alanine if the above hypothesis is correct, and 3 mol. NH_3 would be liberated.

To test this theory alanine was incubated with excess ornithine and the ammonia produced estimated. In preliminary experiments great difficulty was experienced in obtaining significant results owing to the large formation of NH_3 from ornithine itself, especially when the bacterial suspension was very active. This is best made clear by an example. In a usual Thunberg tube experiment the following results were obtained after various times of incubation, the no-substrate control having been deducted:

	NH_3 formed as ml. $N/100$		
Substrate	3·5 hours	10·5 hours	24 hours
(1) Ornithine	0·78	1·30	1·80
(2) Alanine	0	0	0
(3) Alanine + ornithine	3·13	6·09	6·21
(4) Alanine + ornithine minus (1) and (2)	2·35	4·79	4·41

If the working hypothesis outlined above is correct, 6 ml. $N/100$ extra NH_3 (4) should have been formed. Actually the extra NH_3 at first increased with time and then decreased. This decrease is due to the increase in the ornithine blank (1), since the total NH_3 from ornithine and alanine (3) increased throughout. It will be seen that the final ornithine blank (1·8 ml. $N/100$ NH_3) was greater than the NH_3 equivalent of the ornithine added in excess of 2 mol. (1 ml. $N/100$ NH_3). A significant result was therefore scarcely to be expected. Before describing similar experiments in which a larger excess of ornithine was added and which gave significant results it will be convenient to discuss the question of the ornithine blank in more detail.

Ornithine, although deaminated to a certain extent when incubated alone with the organism, can also react with alanine. The question arises as to whether the former type of breakdown occurs concurrently with the latter when ornithine-alanine mixtures are treated with *Cl. sporogenes*, or whether the ornithine-alanine oxido-reduction reaction takes place preferentially. In order to test this 1 mol. alanine was treated with exactly 2 mol. ornithine, i.e. the amount required for complete oxidation of the alanine according to the hypothesis. Sets of Thunberg tubes contained (*a*) water, (*b*) 0·2 ml. $M/10$ alanine, (*c*) 0·4 ml. $M/10$ ornithine, (*d*) 0·2 ml. $M/10$ alanine + 0·4 ml. $M/10$ ornithine, together with 0·5 ml. buffer *p*H 7, 1 ml. bacterial suspension and water to 2·4 ml. Sets of tubes were taken down at intervals and the experiment continued until the NH_3 in (*d*) did not increase. The results obtained are summarized in Table III. It will be seen that, assuming the hypothesis to be correct, the ornithine-alanine reaction is almost complete and all the alanine is broken down as well as the ornithine—in spite of the fact that ornithine alone is more than 50 % deaminated (column 2) and that there was only just sufficient ornithine present to oxidize the alanine.

Table III.

(All values less the appropriate controls.)

	NH_3 formed as ml. $N/100$			
Exp.	Ornithine alone	Ornithine + alanine	Ornithine + alanine (theoretical)	% found of theoretical
1	2·64	5·62	6·00	94
2	2·38	5·50	6·00	92
3	2·76	5·88	6·00	98

The conclusion seems legitimate that ornithine reacts with alanine preferentially to being decomposed alone and that, provided that ornithine is present in only just sufficient quantity to oxidize the alanine, the only reaction to occur will be that oxidation.

If, however, the working hypothesis were incorrect, and ornithine were deaminated at both amino-groups, then the figures for alanine + ornithine in Table III might be due in part to the ornithine blank and not represent a complete oxidation of alanine. This is unlikely in view of the close approximation of the figures to the theoretical, and also in view of the fact that the complete oxidation of alanine was proved by estimation of volatile acid as well as NH_3. Large (75 ml.) Thunberg tubes were set up containing (*a*) 12 ml. water, (*b*) 8 ml. $M/10$ ornithine, (*c*) 8 ml. $M/10$ ornithine + 4 ml. $M/10$ alanine, together with 8 ml. buffer *p*H 7, 8 ml. bacterial suspension and water to 28 ml. The course of the reaction was followed from NH_3 determinations on a series of small tubes containing 1/20 of the above quantities. When these showed the reaction to be complete the large tubes were taken down and NH_3 estimated in duplicate on 1 ml. samples. The remainder of the suspension was used for the estimation (in duplicate) of volatile acid as described by Stickland [1935, 2]. The mean results obtained are given in Table IV.

Table IV.

(All values less the water control.)

	Ornithine alone	Ornithine + alanine	Ornithine + alanine (theoretical)	% found of theoretical
NH_3 produced as ml. $N/10$	1·60	11·43	12·00	95
Volatile acid formed as ml. $N/10$	0	3·63	4·00	91

Assuming, according to the hypothesis, that 2 mol. NH_3 come from the reduction of 2 mol. ornithine and 1 mol. NH_3 from the oxidation of alanine, then from Table IV, the NH_3 equivalent to the oxidation of alanine is $11{\cdot}43/3 = 3{\cdot}81$ ml. $N/10$. Since Stickland [1935, 2] has shown that 1 mol. alanine gives 1 mol. NH_3 and 1 mol. acetic acid, the volatile acid to be expected should therefore be equal to the NH_3, i.e. 3·81 ml. $N/10$; 3·63 ml. $N/10$ or 95 % of that expected was found. It is unlikely that ornithine gives any volatile acid on reduction; if this were so a much higher figure than that obtained would be expected as ornithine is in excess. So that in spite of the blank formation of NH_3 when ornithine is incubated alone with the organism, 2 mol. ornithine were sufficient to bring about the formation of theoretical quantities of both NH_3 and volatile acid from 1 mol. alanine. The reaction between alanine and ornithine undoubtedly takes place preferentially to the breakdown of ornithine alone. The experiments of Table IV also provide strong evidence in favour of the hypothesis concerning the mode of reduction of ornithine, for the results predicted from it are exactly obtained.

One further point in connexion with the ornithine blank is of interest. The question arises as to whether this breakdown is due (*a*) to the decomposition of ornithine alone by *Cl. sporogenes*, or (*b*) to linked reactions with donators derived from the autolysis of the suspensions. In Table IV there was no formation of volatile acid from ornithine alone in the presence of the suspension, and as the chief donators are known to produce volatile acid on oxidation [Stickland, 1935, 2; Author, unpublished] (*b*) is unlikely. This view was confirmed by experiments in which four different concentrations of ornithine were incubated with the same suspension. Since the donators available from autolysis must be strictly limited in amount it would be expected that increase in ornithine concentration would not increase the ornithine blank if (*b*) is correct. The following table shows however that it does increase:

Ornithine	NH_3 formed as ml. *N*/100
0·3 ml. *M*/10	1·88
0·4 ml. *M*/10	2·35
0·6 ml. *M*/10	2·95
0·8 ml. *M*/10	3·45

The evidence is therefore in favour of the partial breakdown of ornithine by a method distinct from linked oxido-reduction reactions.

H equivalence of ornithine. With more detailed knowledge concerning the ornithine blank available it was now possible to design experiments to determine the number of equivalents of H accepted by ornithine during its reduction in the presence of H donators. In order to obtain significant results from NH_3 formation from mixtures of alanine with excess ornithine, it is essential that the ornithine blank shall not exceed the excess ornithine above that required by the hypothesis. In the following experiments twice the theoretical amount of ornithine was used and the ornithine blank in no case exceeded the excess. Sets of Thunberg tubes were set up containing (*a*) water, (*b*) 0·2 ml. *M*/10 alanine, (*c*) 0·8 ml. *M*/10 ornithine and (*d*) 0·2 ml. *M*/10 alanine + 0·8 ml. *M*/10 ornithine; in addition each tube contained 0·5 ml. buffer *p*H 7, 1 ml. bacterial suspension and water to 2·5 ml. Sets of tubes were removed at intervals until the NH_3 in (*d*) no longer increased. The final results obtained in three typical experiments are given in Table V. The value (5) was calculated in the following way. Using the figures of Exp. 3, there were originally present 2 ml. *M*/100 alanine; of this 0·21 ml. has disappeared by blank deamination of alanine (1), leaving 1·79 ml. for interaction with ornithine. Assuming that 1 mol. alanine reacts with 2 mol. ornithine yielding 3 mol. NH_3, then the extra NH_3 formed from alanine + ornithine (4) should be $1·79 \times 3 = 5·37$ ml. *N*/100. The agreement between observed and calculated values (6) shows that 1 mol. alanine is oxidized by 2 mol. ornithine. The fact that alanine is oxidized completely in these experiments has already been established by the volatile acid determinations (Table IV). Since 1 mol. alanine donates 4H on oxidation each mol. ornithine must therefore accept 2H on reduction.

Table V.

Substrate	NH_3 formed as ml. *N*/100		
	Exp. 1	Exp. 2	Exp. 3
(1) Alanine	0·26	0	0·21
(2) Ornithine	3·46	1·96	1·94
(3) Ornithine + alanine	8·80	7·48	7·44
(4) Ornithine + alanine minus (1) and (2)	5·08	5·52	5·29
(5) Theoretical NH_3 corresponding to (4)	5·22	6·00	5·37
(6) % found of theoretical	97	92	98·5

Further evidence that 2 mol. ornithine are required is provided by experiments in which 1 and 2 mol. ornithine were made to react with 1 mol. alanine. The results given in Table VI were obtained. The ornithine blank has not been deducted since it has been shown above that the ornithine-alanine reaction is the only one to occur under these conditions (no excess of ornithine). The theoretical values are those required if 1 mol. alanine reacts with 2 mol. ornithine. 1 mol. ornithine (1) is insufficient to oxidize 1 mol. alanine; it does in fact oxidize almost exactly ½ mol.

Table VI.

Substrate	NH$_3$ formed as ml. $N/100$ Found	Theoretical	% found of theoretical
(1) 0·3 ml. $M/10$ alanine + 0·3 ml. $M/10$ ornithine	4·35	4·50	96·6
(2) 0·3 ml. $M/10$ alanine + 0·6 ml. $M/10$ ornithine	8·61	9·00	95·7

Throughout the experiments so far described concerning the ornithine-alanine reaction the NH$_3$ formed has never exceeded that corresponding to deamination of the alanine and one amino-group only of the ornithine. That one amino-group of ornithine is left unchanged was proved by experiments in which disappearance of amino-N was estimated concurrently with the appearance of NH$_3$. Thunberg tubes were set up as described for the experiments of Table V except that only 0·4 ml. $M/10$ ornithine was used. The residue in the Kjeldahl flask after the distillation of NH$_3$ was quantitatively removed and 2 ml. 30 % trichloroacetic acid added to precipitate the bacteria. After filtration and washing, the filtrate and washings were amalgamated, neutralized with 30 % KOH, made slightly acid with acetic acid and made up to 25 ml. with water. This solution was used for the estimation (in duplicate) of amino-N by the Van Slyke manometric method, 30 min. shaking being employed. The ornithine blank was ignored since only just sufficient ornithine to oxidize the alanine had been present (see above). Estimations were also done before incubation to obtain the amino-N originally present. The results given in Table VII were obtained after incubation until the NH$_3$ determinations showed that the reaction was approximately complete; the water blank has been deducted in each case. These results show (6) that 50 % of the amino-N of ornithine remains unchanged at the end of the reaction, although alanine is completely deaminated. The fact that the amino-N disappearing closely approximates to the ammonia-N appearing, (4) and (5), provides confirmation of the accuracy of the experiments.

Table VII.

	Exp. 1	Exp. 2
(1) Ornithine amino-N originally present (mg.)	1·054	1·002
(2) Total amino-N originally present (mg.)	1·317	1·282
(3) Total amino-N finally present (mg.)	0·540	0·480
(4) Loss of amino-N (mg.)	0·777	0·802
(5) Gain in ammonia-N (mg.)	0·740	0·792
(6) % final amino-N of (1)	51	48
(7) % completion of reaction (from NH$_3$ estimations)	88	94

Isolation of δ-aminovaleric acid. Evidence has been presented that ornithine takes up 2H and loses one amino-group on reduction. The question as to whether it is the α- or the δ-amino-group which is deaminated could only be finally settled by isolation of the product of the reduction. A large Thunberg tube was put up containing 4 ml. $M/10$ alanine, 8 ml. $M/10$ ornithine, 8 ml. $M/5$ buffer *p*H 7 and 8 ml. bacterial suspension (twice normal strength). The course of the reaction was followed by NH$_3$ determinations on a series of small tubes. After 29 hours these indicated that the reaction was 93 % complete. The large tube was then taken down and made strongly acid with HCl. It was then possible to isolate δ-aminovaleric acid as the α-naphthyl*iso*cyanate derivative by the procedure given by Stickland [1935, 1]. Since less product was to be expected only 0·2 g. α-naphthyl*iso*cyanate was used. The dinaphthylurea formed as bye-product was not completely precipitated in 1 hour so the mixture was allowed to stand overnight at this stage. The crude yield of α-naphthylcarbamido-*n*-valeric acid was 93 mg., equivalent to 47 % of the ornithine used.

The crude product was recrystallized three times from 50 % alcohol. The substance then melted with decomposition at 192° (uncorr.) and the solidified decomposition product melted at 231°. The corresponding figures obtained by Stickland [1935, 1] were 191 and 231° respectively, whilst Keil & Gunther [1933] record 197 and 231°. The substance gave the following analytical data (Weiler):

	Calculated for α-naphthylcarbamido-*n*-valeric acid	Found
C %	67·13	67·10
H %	6·29	6·47
N %	9·79	10·08

It may be concluded that when ornithine is reduced in these reactions it is the α-amino-group which is deaminated. All the experimental evidence obtained agrees with the original hypothesis that when ornithine reacts with H-donating amino-acids in the presence of *Cl. sporogenes* it undergoes reductive deamination at the α-amino-group with formation of δ-aminovaleric acid:

$$CH_2NH_2.CH_2.CH_2.CHNH_2.COOH + 2H = CH_2NH_2.CH_2.CH_2.CH_2.COOH + NH_3.$$

Stereochemical specificity of ornithine. All the experiments so far described have been done with *d*-ornithine. In order to test the optical specificity of ornithine in these linked reactions, the activity of synthetic *dl*-ornithine[1] as acceptor was compared with that of the *d*-form. The NH_3 formation, in the presence of the organism, from 1 mol. alanine and 2 mol. of either *d*- or *dl*-ornithine was estimated after various times together with the usual controls. Table VIII shows that *dl*-ornithine gives precisely similar quantitative results to *d*-ornithine, and that 2 mol. *dl*-ornithine are able to oxidize completely 1 mol. alanine (theoretical NH_3, 6 ml. $N/100$; found, 5·80 ml.). It is obvious therefore that *l*-ornithine as well as *d*-ornithine must act as H acceptor in the "Stickland reaction". Table IX shows that the same lack of optical specificity is true also of the acceptor proline, for *dl*-proline gives similar quantitative results to the natural *l*-form, and 2 mol. *dl*-proline (as with *l*-proline) are sufficient to oxidize completely 1 mol. alanine (theoretical NH_3, 5 ml. $N/100$; found, 4·76).

Table VIII.

	NH_3 formed as ml. $N/100$			
Time	5 hours		18 hours	
Form of ornithine ...	*d*-	*dl*-	*d*-	*dl*-
Substrate				
Ornithine alone	1·64	1·55	2·56	2·59
Ornithine + alanine	4·42	4·32	5·78	5·80

Quantities used: ornithine, 0·4 ml. $M/10$, alanine 0·2 ml. $M/10$.

Since glycine is optically inactive, it is interesting to note that *Cl. sporogenes* shows no stereochemical specificity toward the three amino-acids, glycine, proline and ornithine, which it activates as H acceptors. With the H donators on the other hand Stickland [1934] has found the unnatural *l*-alanine to be inactive.

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[1] I am indebted to Dr H. A. Krebs for the gift of a sample of synthetic *dl*-ornithine.

Table IX.

	NH$_3$ formed as ml. $N/100$			
Time	3 hours		6 hours	
Form of proline ...	*l*-	*dl*-	*l*-	*dl*-
Substrate				
Proline alone	0	0	0	0
Proline + alanine	3·62	3·62	4·81	4·76

Quantities used: alanine, 0·5 ml. $M/10$; proline, 0·5 ml. $M/5$.

Experiments with d-*arginine.*

In view of the positive results obtained with ornithine it was decided to reinvestigate the action of *Cl. sporogenes* on arginine, which like ornithine is a δ-substituted derivative of α-aminovaleric acid. Stickland [1934] has found that arginine was not attacked, but Bessey & King [1934] reported that this amino-acid was completely deaminated by washed cells of *Cl. sporogenes*. The action of washed suspensions of the organism on arginine was tested in the usual way by estimating NH$_3$ formed from the amino-acid alone and in admixture with alanine or proline. The results of two typical experiments are shown in Table X. The source of arginine in Exp. 1 was *d*-arginine hydrochloride, and in Exp. 2 the free base; both solutions were neutralized before use. It will be seen that arginine is considerably deaminated when incubated alone with the bacterial suspension (1). The NH$_3$ formed approaches 3 mol. per mol. arginine; this would require the formation of 15 ml. $N/100$ NH$_3$. It seems probable that three of the four amino- and imino-groups of arginine are deaminated. But in addition to this breakdown alone, extra NH$_3$ beyond the controls was found in the presence of alanine (7) proving that arginine also acts as H acceptor. There was no extra NH$_3$ in the presence of proline (5) showing that arginine does not act as H donator.

Table X.

	NH$_3$ formed as ml. $N/100$	
Substrate	Exp. 1	Exp. 2
(1) Arginine	14·19	13·01
(2) Alanine	0·27	0·16
(3) Proline	0	—
(4) Arginine + proline	13·94	—
(5) Arginine + proline minus (1) and (3)	0	—
(6) Arginine + alanine	17·42	18·09
(7) Arginine + alanine minus (1) and (2)	2·96	4·92
(8) Arginine + alanine (no bacterial suspension)	0	0

Quantities used: 0·5 ml. $M/10$ solution of each substrate.

The activation of arginine as H acceptor by *Cl. sporogenes* was confirmed by experiments on the reoxidation of benzylviologen (as described for ornithine):

Substrate	Reoxidation time
Water	Not reoxidized
d-Arginine	3 min.
l-Proline	3 min.

Arginine is therefore able to accept H from reduced benzylviologen. Methylene blue reduction experiments confirmed that arginine has no activity as H donator.

At first sight it seemed possible that arginine might be broken down to ornithine and urea by an arginase enzyme and that the ornithine liberated then acted in the usual way as H acceptor. But the amount of NH_3 actually produced from arginine could only agree with such a theory if the urea formed were also deaminated by the bacterium. Preliminary experiments have shown that this is not the case, for urea was not attacked either alone or in admixture with donators or acceptors. Further work on the arginine problem is in progress.

DISCUSSION.

Fildes & Richardson [1935] obtained slight growth of *Cl. sporogenes* on amino-acid media from which all Stickland's acceptors (glycine, proline and hydroxyproline) had been excluded. These workers considered that the energy for growth in this case might have been derived from serine or tyrosine. In the present paper it has been shown that two other H acceptors, ornithine and arginine, exist in addition to those named by Stickland [1934]. As the medium of Fildes & Richardson contained arginine it seems possible that linked reactions between donators and this acceptor might provide the energy for the slight growth mentioned above.

If the action of *Cl. sporogenes* on amino-acids, as far as is at present known, is considered from the point of view of the relation between the structure of the amino-acids and the method by which they are attacked by the organism, several interesting points emerge. With regard to the method of attack the amino-acids may be divided into two main groups:

(1) Amino-acids which are activated as H donators. This group may be further divided into two sections:

(*a*) Those amino-acids such as alanine, leucine, valine etc. which are attacked only in the presence of an H acceptor. These acids are characteristic simple α-monoamino-acids.

(*b*) Amino-acids which, in addition to acting as H donators, can be partly deaminated in the absence of H acceptors. The two examples are cysteine and serine; these are characterized by the presence of a second substituent group which is not an amino-group in the molecule.

(2) Amino-acids which are activated as H acceptors. This group may also be divided into two sections:

(*a*) Amino-acids which act only as H acceptors and are not attacked in the absence of an H donator. The two examples are proline and hydroxyproline; these are not true amino-acids but imino-acids having ring closure through the amino-group.

(*b*) Amino-acids acting as H acceptors but also partially deaminated in the absence of H donators. Such acids are glycine, ornithine and arginine. Glycine is an abnormal amino-acid as being the first member of the homologous series; it is for example optically inactive. Arginine and ornithine are characterized by the presence of further amino-groups in the molecule in addition to the α-group.

From this list a few general conclusions may be drawn. Simple α-monoamino-acids, with the exception of glycine which behaves abnormally, appear to act only as H donators. When there is another substituent group in the chain in addition to the α-amino-group then the amino-acids behave abnormally and are broken down by *Cl. sporogenes* in the absence of any other amino-acid. If this extra group is not an amino-group, as in cysteine and serine, the substance also acts as H donator; on the other hand if the extra group is an amino-group or contains an amino-group, as with ornithine and arginine, then the substance also acts as H acceptor. It should be emphasized that these considerations apply only to amino-acids which are known to be attacked in one way or other by *Cl. sporogenes*. The structure of lysine, for example, would place it in group 2 *b*; however, lysine is not attacked by the organism in any way [Stickland, 1934]. All that can be said at the present time is that there does seem to be some relationship between the structure of the amino-acids and their mode of activation by *Cl. sporogenes*. It is hoped that such considerations may eventually lead to a clearer understanding of the mechanism of these linked oxidation-reduction reactions.

SUMMARY.

The work of Stickland on coupled reactions between pairs of amino-acids induced by *Cl. sporogenes* has been continued. The following new facts emerged:

1. *l*-Cysteine acts as H donator in these reactions; in addition it is partially deaminated in the absence of other amino-acids.

2. *d*-Arginine and *d*-ornithine are both activated as H acceptors, but in addition are partially deaminated in the absence of H donators. When ornithine reacts with the donator alanine it accepts 2H and undergoes reductive deamination to δ-aminovaleric acid.

3. Glycollic acid, β-alanine and taurine are not attacked by the organism.

The relation between the structure of the amino-acids and their mode of activation by *Cl. sporogenes* is discussed.

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CCLXXII. THE PRODUCTION OF CITRIC ACID FROM LACTIC ACID AND FROM ALCOHOL.

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In the dairy industry moulds are often encountered on media containing lactic acid. According to the literature on the subject, the view appears to be justified that this acid is oxidized by the moulds into CO_2 or transformed into oxalic acid. Wehmer [1929] reports that by the action of *Aspergillus mutantus* calcium lactate is transformed into carbonate and sodium lactate into oxalate. Similarly, Thies [1934] finds that *A. fumigatus* oxidizes calcium lactate to calcium carbonate, whilst its sodium, potassium and ammonium salts are changed into the oxalates of the respective cations. Acklin [1929] states that *Penicillium glaucum* oxidizes lactic acid to water and carbon dioxide via pyruvic acid and acetaldehyde, in agreement with Neuberg's theory. According to Walker & Coppock [1928] *A. niger* acting on calcium propionate first forms lactic acid which is then transformed into pyruvic acid; propionic acid therefore would appear before the lactic acid. These authors accept the view that acetic acid must also be formed as a transient product by dismutation of acetaldehyde, the presence of which was determined by them in these processes.

Botrytis cinerea is a mould which relatively easily transforms acetic into citric acid [Chrząszcz & Zakomorny, 1936]. If then acetic acid is formed during the decomposition of lactic acid, this would indicate that conditions exist for the relevant mould to form citric acid. The determination of this fact would afford further evidence in support of our hypothesis that citric acid is yielded by acetic acid [Chrząszcz & Tiukow, 1930], whilst this would in addition throw rather a different light upon the problem of the decomposition of lactic acid by moulds. The following research was undertaken in order to elucidate this point.

EXPERIMENTAL.

The spores of the mould *B. cinerea* were inoculated into four or five 750 ml. flasks each containing 100 ml. of 8 % sterilized malt extract. After allowing the mould to develop during 10 days at 22°, the extract was decanted, the mycelium was carefully washed with sterilized water, and to each flask 100 ml. of a solution of pure lactate (Merck) free from formic, acetic and propionic acids, were added. The cultures were then kept for a suitable time (see Table I) at 22°, after which they were analysed with the following results.

2·5 % solution of calcium lactate with the addition of 1 % calcium carbonate.

The developed mycelium was kept in a 2·5 % solution of calcium lactate with the addition of 1 % $CaCO_3$. After 12 days the solution was filtered off and the mycelium carefully washed (solution A). The mycelium with the sediment was treated with 20 % acetic acid at room temperature for 24 hours, and then filtered off and washed (solution B). 8 % HCl was then poured on to the mycelium and, after 24 hours, filtered off and the residue washed (solution C).

The washed mycelium was then dried at 105°. It was found to weigh 2·45 g. (computed for five flasks, see Table I).

Solution A was divided into three portions. One was treated with dilute sulphuric acid and distilled. In order to neutralize the distillate, 7 ml. of $N/10$ NaOH were used, thus indicating the presence of volatile acids in the distillate. After evaporating to dryness, a small amount of residue was obtained which upon acidification with sulphuric acid yielded a sharp smell, similar to that of acetic acid, and in addition had the odour of rancid butter. Since the reactions for butyric acid [Kline, 1934] and formic acid were negative, it was concluded that this residue contained a mixture of acetic and propionic acids.

The second portion of solution A was concentrated by evaporation during which process a sediment appeared which gave the reaction for citric acid. After filtering, it was washed, purified with bone charcoal and isolated in the form of a calcium salt, which after drying at 130° weighed 0·627 g.

Analysis. Found: CaO, 33·46 %. $(C_6H_5O_7)_2Ca_3$ requires CaO, 33·74 %.

This result is sufficiently concordant to show that the sediment examined is calcium citrate. If it be postulated that three molecules of lactic acid are necessary for one of citric acid, then to secure 0·627 g. of calcium citrate 0·824 g. of calcium lactate are necessary, and therefore the mould in this case used 8·3 % of the calcium lactate for this purpose.

The solution obtained after filtering off the calcium citrate was brought up to 200 ml. in the flask, and of this volume 5 ml. were used for determining lactic acid by means of a Lieb and Zacherl apparatus [1932]. 11·3 ml. of $N/10$ iodine were required, which, computed for the whole of solution A, gives 90·46 ml. of $N/10$ iodine, equivalent to 4·071 g. of lactic acid or 4·931 g. of calcium lactate. As 10·0 g. of calcium lactate were taken for the experiment, 50·7 % of it had been consumed by the mould.

The remainder of the solution was treated with lead acetate. Only a small precipitate was produced, and this, after decomposition with hydrogen sulphide, yielded a small amount of an acid substance, giving a positive result to Denigès and Stahr's reaction for citric acid. Apart from this, the solution of this salt decolorized a solution of $KMnO_4$ at room temperature and yielded a slight precipitate with a solution of $Hg_2(NO_3)_2$ acidified with nitric acid. The precipitate yielded by the lead acetate consisted of small quantities of lead salts of citric and fumaric acids.

The filtrate from the lead precipitate was treated with ammonia. A precipitate formed, which was washed and decomposed by hydrogen sulphide. The substance obtained gave a distinct reaction for malic acid; with a solution of ferric chloride a deep yellow colour was yielded. The reactions for citric and succinic acids were negative. The substance examined was therefore malic acid.

The third portion of solution A was treated with dinitrophenylhydrazine. The resulting precipitate was washed and treated with N sodium carbonate. 8 % HCl added to the filtrate produced a small precipitate which after washing and drying had M.P. 216°, indicating that it was the hydrazone of pyruvic acid.

The precipitate left after washing with sodium carbonate was treated with ethyl alcohol. A small amount of residue was left on the filter-paper but it was difficult to identify. The alcoholic filtrate on evaporation yielded a small residue, which melted at 164–165°, thus pointing to the presence of the hydrazone of acetaldehyde.

Solution B in acetic acid was evaporated to dryness yielding 6·433 g. of a substance which, computed in terms of calcium acetate, corresponds to 4·06 g.

of $CaCO_3$. Since 4 g. $CaCO_3$ were used, its quantity remained practically unchanged. After dissolving this residue in water, it was treated with lead acetate and then by hydrogen sulphide. The slight amount of material obtained in this manner gave a distinct reaction for malic acid, but negative reactions for citric and succinic acids.

Solution C in HCl after neutralizing with ammonia yielded a white precipitate of calcium salt weighing 1·007 g.

Analysis. Found: CaO, 38·20 %. C_2O_4Ca, H_2O requires CaO, 38·38 %.

0·2024 g. of the substance, required for oxidation 27·6 ml. of $N/10$ $KMnO_4$, corresponding to 0·2016 g. of C_2O_4Ca, H_2O.

The substance was therefore calcium oxalate. If it be assumed that two molecules of lactic acid yield two of oxalic acid, then 1·007 g. of calcium oxalate correspond to 1·504 g. of calcium lactate, or, to produce the above amount of oxalic acid, the mould used up 15·0 % of the calcium lactate.

It follows from the above that the mould used up 50·7 % of the calcium lactate, of which 8·3 + 15·0 = 23·3 % were used for the formation of citric acid and oxalic acid. The remainder (27·4 %) primarily served to form CO_2 and small quantities of malic, fumaric, acetic, propionic and pyruvic acids and acetaldehyde, besides traces of other, unidentified products.

5 % solution of calcium lactate with the addition of 1 % calcium carbonate.

This experiment was conducted as in the preceding series but with the differences that a higher concentration of calcium lactate was used, viz. 5 %, and that the mycelium acted for a longer time, i.e. for 18 days. The results attained were, however, qualitatively identical, only the quantitative relation being different. The mould used up 43 % of the calcium lactate during the experiment, 12·7 of which were used for the formation of volatile acids and citric and oxalic acids, and 30·3 for the formation of CO_2, the increment of the mycelium and for traces of other products.

5 % solution of ammonium lactate with addition of 3 % calcium carbonate.

The mycelium was kept on ammonium lactate for 21 days. Examination of the liquid showed that the products were almost identical with those obtained from calcium lactate, apart from fumaric acid which was not found. The mycelium used up 55·3 % of the ammonium lactate, of which 13·8 were used for the production of volatile acids and citric and oxalic acids, and 41·5 for CO_2, increment of the mycelium and small quantities of unidentified products.

5 % solution of ammonium lactate without any additions.

The action of the mould was maintained for 18 days. The products of decomposition were similar to those obtained in the previous cases, apart from oxalic acid which was not found. The consumption of lactate was greater than before, namely 58·3 %, of which 1·4 appeared as volatile acids and citric acid, and the remainder was used chiefly for production of CO_2 and of small quantities of unidentified products.

2·7 % ethyl alcohol with the addition of 4 % $CaCO_3$.

In order to get a better idea of the behaviour of the mould *B. cinerea* an experiment with ethyl alcohol was made. With this in view, the mycelium was grown on a 2·7 % solution of ethyl alcohol to which 4 % $CaCO_3$ had been added. After 18 days the fluid was filtered and solutions prepared as before: A an aqueous solution, B in acetic acid, and C in HCl.

Part of solution A was distilled. The distillate had an alcohol content of 0·4° Tralles, which, computed for the quantity of solution, shows that the mould used up 90·74 % of the alcohol.

During the evaporation of the solution, a precipitate (0·279 g.) was formed which after purifying gave a reaction for citric acid. Lead acetate was then added to the solution and, after decomposing the lead salt, citric acid was obtained. Thus the aqueous solution yielded 0·279+0·192=0·471 g. citric acid.

In the filtrate a precipitate was produced by ammonia which yielded malic acid. It weighed 0·039 g. and, assuming that one molecule of malic acid is formed from two of ethyl alcohol, the mould used up 0·036 % of alcohol to form this amount of malic acid. The presence of fumaric and succinic acids was not noted.

The second portion of solution A was acidified with sulphuric acid and steam-distilled. The distillate was neutralized and evaporated to 100 ml., and formic acid was determined in 40 ml. by the method of Fincke [Abderhalden, 1925]; the amount found was 0·0112 g. Assuming that one molecule of formic acid was formed from one of alcohol it appears that 0·131 % of the ethyl alcohol was required.

A residue of barium acetate (0·0315 g.) was obtained by adding baryta water to a second 40 ml. of the distillate and evaporating. It was calculated that the whole solution therefore contained 0·073 g. of acetic acid, which indicates that 0·84 % of the alcohol was used for its production.

Lead acetate added to solution B produced a precipitate from which 0·256 g. of citric acid was obtained. In all, the following amounts of citric acid were obtained: 0·471 g. from solution A+0·256 from solution B=0·727 g. of the acid. Taking one molecule of citric acid for three of ethyl alcohol, 0·511 g. of the latter was needed, namely, in our experiment, 5·947 % of ethyl alcohol.

After neutralizing solution C with ammonia, a precipitate was formed which was found to be calcium oxalate weighing 0·215 g. Assuming that for one molecule of oxalic acid one of alcohol is needed, the mould used 0·795 g. of the ethyl alcohol for its production.

Table I. *Results of action of* B. cinerea.

		Mycelium	% of substrate used by mould to form											
Substrate	Days	Wt. for five flasks g.	Formic acid %	Acetic acid %	Propionic acid %	Succinic acid %	Fumaric acid %	Malic acid %	Citric acid %	Oxalic acid %	Pyruvic acid %	Acetaldehyde %	CO_2 and other products %	Total %
2·5% Ca lactate + 1% $CaCO_3$	12	3·06	0·0	Traces	Traces	0·0	Small amount	Small amount	8·3	15·0	Small amount	Small amount	27·4	50·7
5% Ca lactate + 1% $CaCO_3$	18	2·77	0·0	0·6		0·0	Traces	,,	4·1	8·0	,,	,,	30·3	43·0
5% NH_4 lactate + 3% $CaCO_3$	21	2·48	0·0	0·9		0·0	0·0	,,	4·0	7·9	,,	,,	41·5	55·3
5% NH_4 lactate alone	18	2·92	0·0	0·6		0·0	0·0	,,	0·8	0·0	,,	,,	56·9	58·3
2·7% ethyl alcohol + 4% $CaCO_3$	18	5·49	0·131	0·84	0·0	0·0	0·0	0·036	5·95	0·79	—	—	82·9	90·7

The total consumption of alcohol by the mould was 90·7 %, of which 5·947 % was used for the citric acid, 0·036 % for the malic acid, 0·131 % for the formic acid, 0·84 % for the acetic acid, and 0·795 % for the oxalic acid. The remainder, i.e. 82·95 % of the alcohol, was utilized chiefly for the formation of CO_2 and small amounts of unidentified products.

DISCUSSION.

The object of the experiment was primarily to draw attention to the behaviour of *B. cinerea* upon ethyl alcohol as a medium. The alcohol was found to be a good medium since over 90 % of it was consumed, of which over 80 % was oxidized. This oxidation leads by the simplest route to CO_2 as is shown by the presence of acetic and formic acids.

The observation that acetic, malic and citric acids are found on the ethyl alcohol medium, confirms our previous researches on the formation of citric acid from ethyl alcohol [Chrząszcz *et al.* 1932].

If we now examine the behaviour of *B. cinerea* on lactate we are first of all struck by the fact that in every case examined during our experiments citric acid is formed from lactic acid. The formation of acetic, fumaric and malic acids from lactic acid on the one hand confirms our view that citric acid is formed from acetic acid [Chrząszcz & Tiukow, 1930; Chrząszcz & Zakomorny, 1936], and on the other hand indicates that the views of Wehmer [1929], Thies [1934], and Acklin [1929], that lactic acid under the influence of moulds undergoes transformation only into CO_2 or into oxalic acid, are incorrect. It is also of interest to note that ammonium lactate alone, which Thies states is transformed into oxalic acid by the action of moulds, in our experiments yielded absolutely none of this acid, which, however, appeared on the addition of excess $CaCO_3$.

Propionic acid is formed from lactic acid by way of acetaldehyde, as explained by the following reactions, which also cover the formation of the other products:

(*a*) $CH_3.CHOH.COOH-2H\rightarrow CH_3.CO.COOH.$

(*b*) $CH_3.CO.COOH\rightarrow CH_3.CHO+CO_2.$

(*c*) $\left.\begin{array}{l}CH_3.CHOH.COOH\\ CH_3.CHO\end{array}\right\}\rightarrow\begin{array}{l}CH_3.CH_2COOH\\ CH_3.COOH.\end{array}$

(*d*) $CH_3.CHO\rightarrow CH_3COOH.$

(*e*) $$\begin{tikzcd} CH_3.CH_2OH \arrow[r] & CH_3.COOH \arrow[r] \arrow[d] & H.COOH. \arrow[dl, shift left] \arrow[d] \\ & COOH.COOH \arrow[r] \arrow[ur, shift left] & CO_2 \end{tikzcd}$$

(*f*) Acetic acid →succinic acid→fumaric acid→malic acid + acetic acid→citric acid.

It follows from the above that the action of *B. cinerea* on lactates first causes the transformation of lactic acid into pyruvic acid which undergoes decarboxylation forming acetaldehyde. The acetaldehyde is either oxidized to acetic acid, or with a molecule of lactic acid undergoes dismutation into propionic acid and acetic acid.

The acetic acid formed is changed via succinic, fumaric and malic acids into citric acid, or is oxidized via glycollic, oxalic and formic acids into CO_2 and water. It is possible that in older cultures the citric acid formed also undergoes decomposition.

SUMMARY.

1. Citric acid can be formed from lactic acid by the action of the mould *Botrytis cinerea* on lactates.

2. Apart from citric acid, various other products are formed from calcium

lactate, viz. acetic, propionic, fumaric, malic, pyruvic and oxalic acids, acetaldehyde and, in addition, CO_2.

The above compounds are also formed from ammonium lactate, with the addition of calcium carbonate, but the formation of oxalic acid was not observed in the case of ammonium lactate alone without the addition of calcium carbonate.

3. The products yielded indicate that lactic acid passes by dehydrogenation into pyruvic acid, next into acetaldehyde which then undergoes further transformation. With lactic acid, propionic acid and acetic acid are formed by dismutation. Acetic acid is also formed by the direct oxidation of acetaldehyde and changes on the one hand into citric acid, via succinic, fumaric and malic acids, in agreement with our theory, or into CO_2 via formic and oxalic acids on the other hand.

4. *Botrytis cinerea* also easily utilizes ethyl alcohol, transforming it partly into citric acid in agreement with our theory, via glycollic acid and then malic acid. The final oxidation proceeds via formic acid.

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CCLXXIII. A COMPARISON OF SOME METHODS FOR THE DETERMINATION OF BILE ACIDS IN BILE, AND THE PROPORTION BETWEEN THE DIFFERENT ACIDS.

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NUMEROUS methods for the determination of the bile acids in bile have been published utilizing for instance the Pettenkofer reaction, determination of amino-groups in the amino-acid components of the conjugated acids, optical properties, determination of taurine-sulphur, fluorescence with sulphuric acid etc. Each of these methods, however, is limited to the determination of particular bile acids.

We therefore thought it worth while by applying selected methods simultaneously to bile to try to determine the proportion between cholic and deoxycholic, conjugated and unconjugated, glycine and taurine acids. At the same time we have tested, compared and partly modified the various procedures.

Methods

The bile was mixed with alcohol, boiled, diluted to 10 vols. and filtered. Aliquot parts were then evaporated to dryness with some $NaHCO_3$ on the water-bath.

The colorimetric method. As already pointed out by Nakagawa & Fujikawa [1930] and by Reinhold & Wilson [1932], deoxycholic acid and its conjugates do not give the Pettenkofer reaction or its modifications. We have tested some other bile acids with furfuraldehyde and sulphuric acid and found that the following failed to react· cholanic, bilianic, dehydrocholic, chenocholic, α-hyoglycocholic, β-hyoglycocholic acid and "dyslysine";[1] the only positive results were obtained with cholic acid and its conjugates, including scymnol. The reaction was proportional to the molar concentration, whether the substrate was cholic, glycocholic or taurocholic acid and the colour and its absorption spectrum were equal for all these acids. The best way (of many tested) to carry out the reaction proved to be that described by Gregory & Pascoe [1929] as modified by Josephson [1935]. In this form the reaction is sufficiently specific and sensitive for most biles. Cholesterol does not react. The differences between the duplicates from any one bile were less than 1 %.

The gasometric method. Determination of the amino-groups of glycine and taurine after hydrolysis of the conjugated acids, as first described by Schmidt & Dart [1920], does not reveal the presence of the unconjugated acids which, according to Schönheimer *et al.* [1932] may also occur in the bile. For the determination of the conjugated acids the Van Slyke method gave better values than the formaldehyde titration and the colorimetric method of Folin.[2] Hydrolysis of the conjugated acids is always effected with alkali hydroxide, but the concentration of this and the time of heating employed by different workers vary very much. Table I shows our results for the hydrolysis of glyco- and tauro-cholic acids with KOH of various strengths, according to the different investigators. As the table shows, complete hydrolysis both of glyco- and tauro-cholic acids was obtained only according to Jenke & Steinberg [1930], that is with 15 % KOH 12 hours on a boiling water-bath. This was evident not only from amino-N determinations, but also from colorimetric determination of cholic acid in the ether-petroleum extract. Therefore this method was chosen. Even so, however, there often occurred a loss of 1–2 % N. By total N analysis it could always be shown that this loss was due to disappearance of N from the solution, probably as NH_3. In bile this method gave fairly satisfactory values. The results with two or three samples from each bile did not differ more than 1–2 %. Of course the Van Slyke value found before hydrolysis had to be subtracted from that after hydrolysis to obtain the amount of bile acid-N. (With the hypobromite method of Cuny [1930, 1, 2] we did not obtain consistent values.)

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[1] The substances used in these tests were prepared by the late Prof. O. Hammarsten, Uppsala. His successor, Prof. G. Blix, kindly placed them at our disposal.

[2] The statement of Rosenthal & Lauterbach [1924] that taurine with this method should give only 2/3 of its N could not be confirmed by Josephson & Swedin. On the contrary, they found that glycine and taurine gave equimolecular amounts of N.

Table I.

Acid	Conc. of substrate %	KOH %	Hours of heating	NH_2-N mg./ml.		Extracted cholic acid % colorimetric		Extracted cholic acid % polarimetric		Method
				Found	Calc.	Found	Calc.	Found	Calc.	
Glycocholic	6·65	5	6	1·26	2·00	4·71	5·83	4·40	5·83	Sullmann & Schaub [1932], Breusch [1934]
,,	1·66	5	6	0·35	0·50	1·06	1·46	0·89	1·46	
Taurocholic	7·36	5	6	1·92	2·00	5·51	5·83	4·90	5·85	
,,	1·84	5	6	0·45	0·50	1·20	1·46	1·04	1·46	
Glycocholic	6·65	8	8	1·64	2·00	5·24	5·83	5·10	5·83	Foster & Hooper [1915], Rosenthal & v. Falkenhausen [1923], Schmidt & Dart [1920], Smith *et al.* [1928], Cuny [1930, 1, 2], Douglas-Sauermann [1935], Rosenthal & Lauterbach [1924]
,,	1·66	8	8	0·43	0·50	1·25	1·46	1·12	1·46	
Taurocholic	7·36	8	8	1·93	2·00	5·65	5·83	5·55	5·83	
,,	1·84	8	8	0·47	0·50	1·33	1·46	1·20	1·46	
Glycocholic	6·65	15	12	1·96	2·00	5·79	5·83	5·77	5·83	Jenke & Steinberg [1930], Borgatti [1932]
,,	1·66	15	12	0·48	0·50	1·46	1·46	1·42	1·46	
Taurocholic	7·36	15	12	2·02	2·00	5·75	5·83	5·73	5·83	
,,	1·84	15	12	0·51	0·50	1·49	1·46	1·44	1·46	
Cholic	5·00	15	12	0	0	4·94	5·00	4·95	5·00	
Deoxycholic	5·00	15	12	0	0	0	0	4·93	5·00	
Glycine	0·5357	15	12	0·98	1·00	0	0	0	0	
Taurine	0·8929	15	12	1·00	1·00	0	0	0	0	

The polarimetric method. For polarimetric determination of the acids it was also necessary to obtain complete hydrolysis since the reading of a mixture of several acids would not permit any conclusions. According to Jenke & Steinberg [1930] it is also essential to isolate the unconjugated acids after removal of the cholesterol by extraction with a mixture of ether and light petroleum (4 : 1). Using separating funnels for this extraction we very seldom obtained complete extraction. We therefore let the ether mixture bubble through a long narrow funnel in the acidified hydrolysis solution placed in a wide tube in the middle section of an ordinary Soxhlet apparatus. By extracting in this way for 6 hours, during this time making the aqueous fraction alkaline and again acid four or five times, we always obtained such complete extraction that even the furfuraldehyde reaction became negative in the aqueous phase. (It must however be mentioned that this reaction is much less sensitive in strong salt solutions than in weak ones.) The ether then was evaporated and the residue dissolved in absolute alcohol and determined polarimetrically. As Table I shows we got fairly good values with this method in standard solutions. In bile the results with this method were somewhat poorer, since the different samples of one bile often differed by 2–3 % and in some cases even much more as shown in Table II. The loss was probably due to destruction of bile acids.

Attempts to separate only the preformed unconjugated acids from the conjugated ones by such extraction did not succeed since occasionally glycocholic acid was somewhat soluble in the ether mixture and taurocholic slightly so. This is the reason why, as shown in Table I, in the incompletely hydrolysed samples the colorimetric values in the ether extract are usually higher than the polarimetric ones. Results obtained from bile by the polarimetric method must be accepted with reserve as in most biles there are small amounts of bile acids other than cholic and deoxycholic, e.g. ursocholic, hyocholic, lithocholic etc., which are optically active. This is of special importance for human bile which contains anthropodeoxycholic and pig's bile containing hyocholic acid with specific rotations differing greatly from those of cholic and deoxycholic acids. Nevertheless we have tried to get an approximate estimate of deoxycholic acid in some biles by subtracting from the total rotation of the mixture the rotation of the colorimetrically determined cholic acid and then calculating from the specific rotation of the deoxycholic acid. We used wave lengths of 6563 and 5893 Å and sometimes of 6867 Å where the absorption was less when much bile pigment was present. The concentrations are calculated from Josephson's [1935] investigation of the optical properties of the bile acids and the same apparatus was used. In most cases the readings were made in 4 dm. tubes, but in some cases, where the extracts were very dark we had to read in 2 dm. tubes. We also tried to make the polarimetric readings in the alkaline hydrolysate after extraction of cholesterol. This would be possible as there could not be present any other optically active substances to count with.

The specific rotation of the bile salts in water solution is well known but, as shown by Josephson, this rotation decreases with increasing concentration of other electrolytes in the solution. In these cases we therefore had to add exactly known amounts of $NaHCO_3$ (at the stage of evaporation of the alcohol) and KOH. The readings of these solutions, however, often became somewhat inexact, owing to the large amount of light-absorbing bile pigments present. In many biles, especially the human ones, the pigments made the observations in alkaline solution impossible, even if light of wave length 6867 Å was used. Where this method was applicable however it usually gave results which agreed better with those of the other methods than did the results obtained after acid extraction.[1]

Sulphur determinations. The S has been determined and the concentration of taurine-N calculated and subtracted from the total amino-N, in order to distinguish between glyco- and tauro-cholic acids [Cuny, 1930, 1, 2; Douglas-Sauermann, 1935; Rosenthal & von Falkenhausen, 1923]. This method, however, is reliable only in those biles where no S-containing substances other than taurocholic

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[1] Raue [1925], Borgatti [1932], and Giordano & Levi [1933] have tried to determine bile acids with the aid of the fluorescence reaction with sulphuric acid. Josephson & Swedin [unpublished results] have studied this reaction in standard solutions. Using a quartz lamp and the Stufenphotometer of Zeiss with light filter of euphosglass they found that the fluorescence of cholic acid was about ten times as intense as that of deoxycholic acid. The intensity of the fluorescent light was not directly proportional to the concentration of the acid but followed a logarithmic function of this concentration. The intensity was very dependent on other substances, such as inorganic salts, bile pigments, fatty acids etc., which are always present in bile. Since in addition the reaction was not very specific Josephson & Swedin concluded that it could not be used for quantitative determinations.

Table II.

(1) Species	(2) Cholic acid colori-metric millimol	(3) $[\alpha]_D$ of the original bile read in: a Dilution	(3) b Alkaline water	(3) c Alcohol	(4) Calculated α of the cholic acid in the original bile: a Alkaline water	(4) b Alcohol	(5) De-oxycholic acid calc. from $[\alpha]_D$ in water millimol	(6) Sum of cholic and de-oxycholic acids millimol	(7) Con-jugated acid from NH_2-N millimol	(8) Uncon-jugated cholic and de-oxycholic acids millimol	(9) Taurine acids from S deter-mination millimol	(10) Glycine acids from diff. of N and S deter-minations millimol
Cow 1	115	1 : 2·5	1·35°, 1·35°, 1·38°, 1·32°, 1·32°, 1·33°	0·84°, 1·02°, 1·09°	1·36°	1·65°	0	115	70	45	79	Neg.
Cow 2	123	1 : 2·5	0·96°, 1·15°, 1·15°, 1·14°	—	1·44°	1·75°	Neg.	123	82	41	34	48
Cow 3	129	1 : 2·5	1·64°, 1·63°, 1·56°*	—	1·52°	1·84°	5	134	131	3	87	44
Cow 4	112	1 : 2·5	1·52°, 1·52°	—	1·32°	1·60°	10	122	121	1	65	56
Cow 5	101	1 : 2	1·47°, 1·56°, 1·52°, 1·49°	—	1·20°	1·44°	31	132	150	Neg.	62	88
Pig 1	0	1 : 2·5	0·01°, 0·02°	0·30°, 0·72°	0	0	Trace	Trace	118	Neg.	21	97
Pig 2	0	1 : 2·5	0·06°, 0·08°, 0·18	0·30°, 0·30°	0	0	Trace	Trace	121	Neg.	23	98
Pig 3	0	1 : 2·5	0·09°, 0·01°, 0·11°	—	0	0	Trace	Trace	170	Neg.	19	151
Sheep 1	175	1 : 4·17	2·23°, 2·24°	—	2·03°	2·52°	26	201	185	16	209	Neg.
Sheep 2	266	1 : 2·33	2·65°, 2·56°	1·96°, 2·07°, 1·70°	3·05°	3·79°	Neg.	266	305	Neg.	219	86
Rabbit	55	1 : 12·82 1 : 5·13	3·91° 4·07°	—	0·68°	0·81°	241	296	152	144	111	141
Cat	337	1 : 7·25	—	2·52°, 3·44°	3·80°	4·81°	Neg.	337	295	42	432	Neg.
Dog	165	1 : 5	2·02°, 1·94°, 1·98°, 1·82°	2·37°, 1·64°	1·92	2·37°	3	168	317	Neg.	193	124
Fox	127	1 : 5·56	1·02°	—	1·49°	1·81°	Neg.	127	39	88	126	Neg.
Guinea-pig 1	5	1 : 2·23	0·00°	—	0·07°	0·07°	0	5	23	Neg.	—	—
Guinea-pig 2	5	1 : 5·36	2·59°	—	0·07°	0·07°	227	232	1	231	—	—
Polar bear	219	1 : 4	—	1·41°, 1·39°, 1·68°	2·52°	3·12°	Neg.	219	195	24	244	Neg.
Walrus	360	1 : 4	—	0·84°	—	4·11°	Neg.	360	286	74	—	—

* Not used for the calculation of average.

acid are present. In other biles these acids would have to be isolated before the S determination could be made. We are working on this isolation, and the results obtained with its aid will be published later. Here we report the results of total S determinations and the concentrations of taurine acids calculated from these values. The S determinations were carried out by the method of Josephson [in course of publication].

RESULTS.

The results which were obtained by using the related methods on different animal biles are shown in Table II. In Table III we report the results from some human biles obtained post mortem. It should be pointed out that we have analysed only gall-bladder biles and since we do not know how much water is resorbed from the gall-bladder we cannot draw any conclusions about the concentration in liver bile. The proportions between the different acids, however, are probably not altered in the gall-bladder. The biles of the rabbit, guinea-pig, dog and cat were collected from several animals; the results for polar bear and walrus biles were obtained with preparations about 50 years old, precipitated with alcohol in unknown proportions, probably 1/4; these are recorded only as curiosities. All the other biles were from single individuals.

Table III.

(1)		(2) Cholic acid colorimetric millimol	(3) $[\alpha]_D$ of the original bile read in		(4) Calculated α of the cholic acid in the original bile	(7) Conjugated acid from NH_2-N millimol	(9) Taurine acids from S determination millimol	(10) Glycine acids from difference of N and S determinations millimol
a Human biles Diagnosis	*b* Age years		*a* Dilution	*c* Alcohol				
Cancer recti	69	25	1 : 2·49	1 27°	0·36°	96	48	48
Nephrosis acuta	39	10	—	—	0·15°	78	41	37
Sepsis	20	66	1 : 7·19	1·80°, 2·52°	0·95°	165	98	67
Encephalitis lethargica	29	49	1 : 4·84	1·97°, 2·09°	0·70°	166	75	91
Agranulocytosis	80	27	1 : 4·41	0·30°, 0·65°, 0·65°	0·38°	41	- -	—
Pneumonia acuta	40	17	1 : 4·50	0·46°, 0·45°, 0·50°	0 24°	80	18	62
Haemorrhagia cerebri	57	34	1 : 3·95	1·37°, 1·35°	0·48°	78	32	46
Carcinoma laryngis	60	45	1 : 3·19	1·08°	0·65°	67	18	49
Vitium organicum cordis	65	61	1 : 4·35	0·90°, 1·00°, 0·65°	0·87°	80	54	26
Carcinoma recti	71	14	1 : 3·00	0·17°, 0·15°, 0·12°	0·19°	3	9	Neg.
Arteriosclerosis	70	50	1 : 2·06	0·80°	0·72°	100	38	62
Nephrosclerosis maligna	22	33	1 : 3·23	1·31°, 1·26°, 1·09°	0·47°	78	67	11
Pneumonia acuta	3	2	1 : 3·14	0·00°, 0·02°	0·03°	0·2	4	0
Agranulocytosis	73	81	1 : 3·13	1·05°, 1·13°	1·16°	129	31	98
Haemorrhagia cerebri	70	23	1 : 2·86	0·28°, 0·28°, 0·41°	0·33°	44	28	16
Sepsis pneumococci	43	14	1 : 3·28	0·31°	0 21°	35	—	—

In the colorimetric, gasometric and S determinations only the averages are recorded since the different samples always agreed very closely. On the other hand all the results of polarimetric readings are noted since they usually differed a good deal from one another. Of several biles unfortunately we could not obtain enough for making duplicate analyses by the polarimetric method which requires considerable amounts of material.

Explanation of the tables.

Column 1 in Table II shows the species of animal from which the biles originated. The cow, sheep, pig and fox biles were from single individuals. The rabbit bile was collected from 20 animals, the cat bile from 4, the dog bile from 4, the guinea-pig bile 1 from 40 and the guinea-pig bile 2 from 30 animals.

In Table III column 1 shows the pathological condition and age of the human subjects from which bile samples were taken.

Column 2 shows the results of the colorimetric determinations of cholic acid including the conjugated as well as the unconjugated acids.

Column 3 contains the results of the polarimetric determinations. 3 *a* shows the dilution of the original bile which was used for the determination. 3 *b* shows the optical activity of the original biles calculated from the rotation observed when the bile acids were dissolved in aqueous alkali. 3 *c* gives the activity of the original bile observed when the acids were dissolved in alcohol.

Column 4 indicates the part of the optical activity of the original bile which could be calculated from its colorimetrically determined content of cholic acids only. The difference between these values in aqueous alkali (*a*) and in alcohol (*b*) depends on the different optical activities of cholic acid in these solvents [Josephson, 1935].

Column 5 shows the content of deoxycholic acids, conjugated as well as unconjugated, in the original bile. These values are obtained by subtracting the calculated rotation of the known amount of cholic acids (column 4) from the rotation observed. In this calculation we have used Josephson's [1935] determinations of the specific rotation of deoxycholic acid. In some cases the total rotation of the bile was even smaller than that calculated from the cholic acid content. In these cases we have written Neg. in this column.

Column 6 contains the sums of cholic and deoxycholic acids, conjugated as well as unconjugated. These values represent the sum of those from columns 2 and 5.

Column 7 shows the content of conjugated acids, glyco- as well as tauro-cholic and deoxycholic. These values are calculated from the conjugated amino-N (Van Slyke).

Column 8 shows the amount of free unconjugated acids in the bile, calculated by subtracting the conjugated acids (column 7) from the total conjugated and unconjugated cholic and deoxycholic acids (column 6). In cases where the last value (6) is smaller than that for the conjugated ones (7) we have written Neg. in this column.

Column 9 shows the part of the cholic (or deoxycholic) acid which is conjugated with taurine if the content of this substance is calculated from the observed S content of the bile.

Column 10 shows the part of the cholic (or deoxycholic) acid which is probably conjugated with glycine. These values are calculated by subtraction of the taurine acids (column 9) from the total conjugated acids (column 7). In cases where the S content was higher than corresponded to the amino-N content, it was naturally impossible to calculate the glycine content and here we have written Neg. in this column.

Discussion.

As the tables show, there is usually a considerable difference between the results obtained with the four different methods. The polarimetric method must be regarded as rather unsuitable for biles, since it gives values which do not agree well either with one another or with those of other methods. This is certainly due to destruction of bile acids during hydrolysis and extraction. In many cases we even observed rotations which were lower than could be calculated from the colorimetrically determined cholic acid. In pig and guinea-pig biles the furfuraldehyde reaction was almost or completely negative, indicating the absence of cholic acid. As certain of the hyocholic acids are laevorotatory it is not surprising that the polarimetric values of these biles are so low.

In those biles (cow 1, 2, rabbit, cat, fox, guinea-pig 2), where the gasometric value was much lower than those obtained colorimetrically and polarimetrically, we conclude that there must be a large proportion of unconjugated bile acids. In those where the gasometric value is higher than the colorimetric (cow 5, sheep 2, dog) on the other hand much deoxycholic acid and other acids must be present which do not react colorimetrically. In some biles, especially dog bile, the gasometric value was much higher than the colorimetric and polarimetric values. This indicates the presence of some laevorotatory substance present, or possibly of a nitrogenous compound soluble in alcohol, hydrolysed by alkalis and not reacting like cholic acid.

The S content of the different animal biles varied within wide limits. In sheep, cat and fox biles it was very high, showing without doubt that S-containing substances other than taurine are present (sulphuric esters). In dog bile S was also high but insufficient to account for the total conjugated acids. Contrary to the claims of other investigators, we can thus state that dog bile contains conjugated acids other than taurocholic.

The human biles were all from autopsies conducted not more than 2–3 hours after death. Immediately after collection the biles were precipitated with alcohol. Cases were deliberately selected which had no hepatic lesion. As Table III shows, there is no uniformity at all in the bile acid contents of these biles. This is true for colorimetric as well as polarimetric and gasometric values; in addition to this the values found with the different methods do not agree with one another. With only two exceptions the gasometric values are much higher than the colorimetric, indicating that there must be large amounts of conjugated acids, which do not react colorimetrically. Unfortunately the polarimetric determination of the human biles had to be carried out after acid extraction, since they all were so heavily pigmented that alkaline readings were impossible. Since anthropodeoxycholic acid is usually present in human bile, we have not been able to calculate the deoxycholic content, as this acid has a much lower optical activity than the other bile acids. The S analyses of human biles have shown that the taurocholic acid content is probably of the same order as that of glycocholic acid. Owing to these facts it seems difficult to obtain diagnostically valuable information from bile acid analysis.

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CCLXXIV. STUDIES ON CAROTENOIDS.[1]

III. AN ISOMERIDE OF LUTEIN ISOLATED FROM THE FURZE (*ULEX EUROPAEUS*).

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WE have isolated from the yellow flowers of the furze a new carotenoid which in its properties is very similar to lutein. It crystallizes from absolute methyl alcohol in stellate groups of pale yellow twinned prisms. The crystals are dichroic and have a strong birefringence. Like lutein this yellow form, which does not contain methyl alcohol of crystallization, changes in contact with the solvent in the course of 2 or 3 days into a red form of metallic brightness containing methyl alcohol, which cannot be distinguished from lutein by the shape of the crystals. The absorption bands in different solvents are the same as those of lutein, in carbon disulphide 508, 475 $m\mu$, in ethyl alcohol 475, 447 $m\mu$, in methyl alcohol 473, 445 $m\mu$. During chromatographic analysis in mixture with lutein on calcium carbonate, the new carotenoid forms a uniform orange-coloured zone, and in this way it cannot be separated from lutein. It shows, however, a marked difference from lutein in its M.P., which for the yellow methyl alcohol-free form was found to be 201–202° (uncorr., in evacuated tube; corr. 205–206°). The red crystals lose methyl alcohol at 120–130° and melt at 199–200° (uncorr.). Pure lutein from *Genista tridentata* melted 10° lower, at 190° (uncorr.).

For the reasons above mentioned we suppose that we are dealing with a stereoisomeric form of lutein. An analysis could not be made because of the small quantity available. The carotenoid is sparingly soluble in cold carbon disulphide, very sparingly in cold ethyl alcohol and still more sparingly in cold methyl alcohol.

The carotenoids of the furze (Ulex europaeus).

We have begun a systematic investigation of the carotenoids of diverse furze and broom species of the family of Leguminosae the preliminary results of which we report in this and the following papers. The yellow flowers of the furze were investigated long ago by Schunck [1903], who demonstrated the presence of a xanthophyll—named by him xanthophyll Y—which is distinguished by a strong blue colour reaction with concentrated hydrochloric acid. Schunck gives a photograph of the absorption spectrum of the xanthophyll as well as the spectrum of the product formed by hydrochloric acid, and from his data one may conclude that he dealt with violaxanthin.

METHODS AND RESULTS.

A preliminary investigation showed that the carotenoids of the furze are a rather complicated mixture. The flowers do not keep well in a fresh state and oxidize with ease. Therefore we have not attempted to separate the yellow

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[1] These investigations are assisted by a grant from the van't Hoff Foundation of the Royal Academy of Sciences, Amsterdam, for which the author expresses his indebtedness.

petals of the flowers from the green ones of the calyx which represent the greater part of the weight of the flowers. The flowers were dried in a current of warm air at 50°. This procedure for a quantity of 25 kg. of fresh flowers required 8 days, and in consequence of this a large part of the carotenoids was already oxidized. The dry flowers weighed 5·5 kg., which corresponds to a weight of the yellow petals of 1·5 kg. They were ground in a mill to a very fine powder. As the yellow petals do not contain free xanthophyll, the powder was extracted three times with light petroleum in a total volume of 60 litres. The yellow solution was concentrated *in vacuo* in a current of carbon dioxide at 35–40° to 1 litre and freed from a very fine precipitate by centrifuging, and 2 litres of absolute ethyl alcohol were then added. After remaining 2 days in the ice-box a colourless wax-like mass had separated which was removed by suction, the residue being washed on the filter with a little ethyl alcohol. The filtrate was evaporated *in vacuo* and the resulting viscous oil kept in the ice-box for several days, where a further quantity of colourless material settled. After centrifuging, the oil was poured into 2·5 litres of pure acetone by which procedure a considerable quantity of colourless material was precipitated which increased during 1 day in the ice-box. After being again centrifuged, the solution was evaporated to dryness *in vacuo*. The residue was a dark coloured viscous oil of characteristic odour and weighed 330 g.

It has proved advantageous for the isolation of the different carotenoids to include in the process of purification at this point a chromatographic analysis with activated aluminium oxide. In this way a separation of the bulk of xanthophyll esters from carotene as well as from the chlorophyll pigments is easily obtained, and the main part of the xanthophylls is obtained in a state of great purity so that after saponification they readily crystallize.

The residue of the distillation was dissolved in 3 litres of light petroleum and shaken with methyl alcohol (80 %) and then with methyl alcohol (90 %) which extracted the small amount of free xanthophyll which had passed into solution from the green leaves of the calyx, as well as some chlorophyll. The solution was then washed with water to free it from the methyl alcohol and dried over sodium sulphate. It was then adsorbed on to activated aluminium oxide in two columns. By washing the adsorbed colouring matter with light petroleum nine different zones were formed. The rather large upper zone contained only colourless material, then followed four narrow green and reddish brown zones of chlorophyll and xanthophyll. Most of the column was filled with a yellow zone of xanthophyll esters below which followed three smaller orange-coloured zones which contained the carotenes. The column was divided into three parts (*a*) the three lowest zones, (*b*) the large uniform one, and (*c*) the four narrow upper zones.

(*a*) *Hydrocarbons.* It is possible to obtain a part of the hydrocarbons of the lower zones directly in a crystalline state by repetition of the chromatographic analysis and elution. It is, however, advantageous to proceed with an alkaline saponification of the solution before further treatment. The solution of the colouring matter obtained by eluting the lower zones of the chromatogram with light petroleum containing 1 % of methyl alcohol was concentrated *in vacuo* at low temperature to 150 ml. To the concentrate were added 25 ml. of a concentrated methyl alcoholic solution of potassium hydroxide, 50 ml. of ethyl alcohol (96 %) and the same volume of absolute ethyl alcohol, and the mixture was shaken in an atmosphere of nitrogen for 8 hours. After standing overnight, 20 ml. of water and 250 ml. of light petroleum were added, the alcoholic layer separated, the light petroleum solution several times shaken with methyl alcohol (90 %), then washed with water and dried over anhydrous sodium sulphate. The solution was then adsorbed on to a column of activated aluminium oxide and washed with light petroleum. Three distinct zones developed:

	Absorption bands in light petroleum (B.P. 80°)* mμ
1. Narrow very sharp red-violet zone	451, 426
2. Broad orange zone	479, 450
3. Diffuse yellow zone	475, 445

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* All spectroscopic measurements were made with a Hilger prism-spectroscope and a copper sulphate-ammonia filter.

Zones 2 and 3 were eluted together with light petroleum containing 1 % of methyl alcohol, and the solution was evaporated to dryness *in vacuo*. The residue was dissolved in 20 ml. of light petroleum and to the solution 80 ml. of absolute ethyl alcohol were added. After several hours carotene began to separate in glistening crystals accompanied by colourless material. The quantity of crystals increased on keeping the solution in the ice-box for 1 day. The precipitate was filtered and freed from the colourless substance by boiling with 100 ml. of absolute ethyl alcohol. From this solution a sterol was obtained in a pure state. The remaining carotene was then twice crystallized from a mixture of benzene-methyl alcohol (1:3). Characteristic crystals of carotene were obtained; M.P. 181–182° (uncorr. in evacuated tube); absorption bands in light petroleum (B.P. 80°) 481, 450 mμ; in CS_2 517, 482 mμ. A separation into the isomerides was not attempted. A yield of 22 mg. was obtained, i.e. 20 % of the total quantity. The rest of the carotene remaining in the mother-liquors could not be obtained in a crystalline state, as the separation of a great quantity of a colourless oil was not possible.

The first zone after elution with light petroleum and methyl alcohol was evaporated to dryness *in vacuo*. The residue was boiled with the purest methyl alcohol, a colourless substance passing into solution. During repeated boiling with the same solvent nothing more went into solution. We have tried to crystallize the colouring matter from different solvents but could not succeed in obtaining it in a pure state. This pigment is a hitherto unknown carotenoid which in its spectroscopic properties agrees with flavoxanthin [Kuhn & Brockmann, 1932]. Absorption bands in CS_2, 478, 450 mμ; in light petroleum, 451, 426 mμ; in methyl alcohol, 448, 422 mμ.

When shaken with light petroleum and methyl alcohol (90 %) the pigment remains completely in the upper layer. In ethereal solution it gives no colour reaction with concentrated hydrochloric acid. By means of chromatographic analysis with aluminium oxide, on which it is strongly adsorbed, the carotenoid can be separated from carotene with the same ease as it can be separated from lutein with calcium carbonate, by which it is scarcely adsorbed. In these properties the carotenoid is very similar to the xanthophylls of the formula $C_{40}H_{56}O$, cryptoxanthin and rubixanthin [Kuhn & Grundmann, 1933; 1934]. It occurs not only in the furze but also in Planchon's furze (*Ulex galli*) and in very small quantity in *Genista tridentata* (see the following paper). In somewhat greater quantity it occurs in the flowers of the wild clover (*Oxalis cernua*), of which we shall make a report later.

(*b*) *Xanthophylls*. The large middle zone of the first chromatographic separation contains the bulk of the xanthophylls in a moderately pure form. We have tried to isolate a uniform crystalline coloured wax but without success. The pigment after elution with light petroleum containing 1 % of methyl alcohol was concentrated *in vacuo* to 100 ml. Then for saponification were added 25 ml. of concentrated methyl alcoholic KOH, 20 ml. of ethyl alcohol (96 %) and a quantity of absolute ethyl alcohol sufficient to make a homogeneous mixture. The liquid was kept in a well-filled and stoppered bottle for 2 days at room temperature. After complete saponification a considerable quantity of water was added and the free xanthophylls were extracted with ether. The ethereal solution was washed several times with water, dried over anhydrous sodium sulphate and concentrated *in vacuo* to 10 ml. 150 mg. of xanthophyll crystallized, which were twice recrystallized from methyl alcohol. 80 mg. of xanthophyll of M.P. 193–194° were obtained; absorption bands in CS_2, 500·5, 468 mμ. With strong hydrochloric acid a deep blue colour develops. As the xanthophyll did not yet seem to be uniform, it was dissolved in benzene, diluted with four times its volume of light petroleum and adsorbed on to a column of calcium carbonate. After prolonged washing with a mixture of benzene-light petroleum, 1:2 and then 1:1, three well-defined zones developed. The upper brown-yellow zone contained violaxanthin, under which there was a narrow lighter yellow zone of taraxanthin [Kuhn & Lederer, 1931] and at a distance of 2 cm. a third orange-yellow zone, from which the isomeride of lutein was isolated.

Violaxanthin. The zone containing this xanthophyll was eluted with pure methyl alcohol and the solution evaporated to dryness *in vacuo*. The residue was crystallized from methyl alcohol, the crystals boiled with light petroleum and then recrystallized from absolute methyl alcohol. We obtained 35 mg. of crystals which formed brown-red needles of M.P. 197° (uncorr., evacuated tube); absorption bands in CS_2, 500·5, 469 mμ; in methyl alcohol 469, 411 mμ.

Taraxanthin. The second zone was eluted with methyl alcohol and evaporated to dryness *in vacuo*. The residue was twice crystallized from the purest methyl alcohol and 15 mg. of long needles grouped in clusters were obtained: M.P. 185–186° (uncorr., in evacuated tube); absorption bands in CS_2, 501, 469 mμ; in absolute ethyl alcohol, 470, 441 mμ; in methyl alcohol, 468, 440 mμ. With strong hydrochloric acid in ethereal solution no coloration occurs.

Isomeric lutein. The solution obtained by eluting the lowest zone of the chromatogram with methyl alcohol was evaporated to dryness *in vacuo* and the residue crystallized from methyl alcohol. 10 mg. of xanthophyll were obtained, which were boiled with light petroleum and then four times recrystallized from purest methyl alcohol. 3 mg. of crystals were obtained which macroscopically have a reddish brown aspect similar to violaxanthin. Under the microscope yellow dichroic crystals with strong birefringence are observed. The substance gives no colour reaction with concentrated hydrochloric acid; M.P. 201–202° (uncorr., in an evacuated tube) (corr. 205–206°). These crystals change in the course of 2 or 3 days in contact with methyl alcohol into a red form, which contains methyl alcohol of crystallisation. Simultaneous comparison of the melting points of the isomeride with pure lutein from *G. tridentata*: lutein M.P. 190° (uncorr.), isomeric lutein (red form) M.P. 199–200° (uncorr.).

(*c*) *Other xanthophylls.* The xanthophylls contained in the narrow upper zones of the first chromatogram in mixture with the chlorophyll pigments were eluted with light petroleum containing 1 % of methyl alcohol and the solution concentrated *in vacuo* to 20 ml. The concentrate was then saponified with methyl alcoholic KOH and absolute ethyl alcohol sufficient to make a homogeneous mixture. After adding water, the free xanthophylls were extracted with ether, and a deep red ethereal solution was obtained, which gave a strong blue colour reaction with concentrated hydrochloric acid. The solution was washed with water and then evaporated to dryness *in vacuo*. The residue was dissolved in benzene, diluted with the same volume of light petroleum and adsorbed on to a column of calcium carbonate. By washing the chromatogram with a mixture of benzene and light petroleum 1 : 1 several zones developed, which have a spectrum similar to violaxanthin.

	Absorption bands in (B.P. 80°)	
	Light petroleum mμ	Ether mμ
1. Large yellow zone	465, 436	—
2. Small green-yellow zone	466, 436	466, 440, 420
3. Very large lemon-coloured zone	468, 438	465, 440
4. Yellow-brown zone	470, 440	(Violaxanthin)

We have tried without success to crystallize the xanthophylls from these zones. From all solvents only deep red oils separated, which solidified in the cold and became liquid again at room temperature. We suppose that those xanthophylls which are rich in oxygen are unstable to alcoholic KOH and that they lose the power of crystallization by this treatment like fucoxanthin [Willstätter & Page, 1914], or that they are altered in the course of chromatographic analysis with aluminium oxide or calcium carbonate.

The carotenoids of Planchon's furze (Ulex galli).

Planchon's furze differs from *Ulex europaeus* by being smaller in height and especially by having much smaller flowers. The investigation of the pigment of the flowers showed that it consists of the same carotenoids as that of the flowers of the furze. In the case of Planchon's furze carotene is isolated much more easily, as only few colourless substances are present in that fraction. We have also separated the carotene obtained into the isomeric forms and have obtained α- and β-carotene in a pure state.

β-Carotene: M.P. 181–182° (uncorr., in evacuated tube); absorption bands in CS_2, 520, 486 mμ.

α-Carotene: M.P. 185° (uncorr., in evacuated tube); absorption bands in CS_2, 512, 482 mμ; in light petroleum (B.P. 80°), 480, 448 mμ.

In one case of the preparation of the xanthophylls we have observed during chromatographic analysis a small light yellow zone between the zones of violaxanthin and of lutein, from which we extracted a solution of a carotenoid which shows all the properties of flavoxanthin [Kuhn & Brockmann, 1932]. The absorption bands in ethyl alcohol (96 %) are 446, 423 mμ; in CS_2, 475, 452 mμ. With strong hydrochloric acid in ethereal solution a deep blue colour develops. The pigment gives all the colour reactions described by Kuhn & Brockmann for flavoxanthin. We have not, however, succeeded in isolating this carotenoid in a crystalline state.

Some colourless substances accompanying the carotenoids of Ulex galli and U. europaeus.

Hentriacontane. This hydrocarbon was isolated from the mother-liquors of carotene from *Ulex galli*. It separated from the concentrated solution as a light yellow precipitate which was twice crystallized from ethyl alcohol with the addition of a little charcoal. It forms soft leaflets with a silvery lustre which have no birefringence; M.P. 64° (uncorr.).

Micro-analysis (Weiler): Found: C, 85·02 %, H, 14·84 %. $C_{31}H_{64}$ requires C, 85·22 %; H, 14·78 %.

Sterol $C_{30}H_{50}O$. This crystallizes together with carotene from *Ulex europaeus* from a mixture of light petroleum and absolute ethyl alcohol, and can be separated from carotene by boiling with absolute ethyl alcohol. It crystallizes from ethyl alcohol (90%) in nodular masses, from a mixture of acetic ether and methyl alcohol in fine stellate needles; M.P. 152–153° (uncorr.). The sterol is optically inactive, $[\alpha]_D = \pm 10°$. Colour reactions: Salkowski's test; acid light yellow with green fluorescence; chloroform uncoloured: Liebermann-Burchardt test; chloroform intense dark blue; the colour passes into the acid layer, where after some time it turns to violet.

The sterol is perhaps identical with a sterol $C_{30}H_{50}O.\frac{1}{2}H_2O$, isolated from *Gledschia triacanthus* [Dalmer, 1932].

Analysis: For analysis the substance was dried for 2 hours at 60° in a high vacuum, but it still contained water or alcohol of crystallization.

Found: C, 83·11, 83·07; H, 11·88, 11·57%. Mol. wt. (Rast) found 385. $C_{30}H_{50}O, \frac{1}{2}H_2O$ requires C, 82·68; H, 11·72%. Mol. wt. 435. $C_{30}H_{50}O, \frac{1}{2}C_2H_5OH$ requires C, 82·77; H, 11·89%. Mol. wt. 449.

Acetate. The acetate was prepared by boiling the sterol with acetic anhydride. It crystallizes from a mixture of ether and methyl alcohol in rectangular leaflets or brilliant needles, which show a strong birefringence; M.P. 145–146° (uncorr.), the substance softening at 138°. The acetate is, like the sterol, optically inactive.

Analysis: Found: C, 81·96; H, 11·30%. Mol. wt. (Rast) 434. $C_{32}H_{52}O_2$ requires C, 81·96; H, 11·21%. Mol. wt. 468.

Sitosterol. This sterol was isolated from the xanthophyll fraction of *Ulex europaeus*. After two crystallizations from methyl alcohol it forms leaflets grouped in rosettes with strong birefringence. $[\alpha]_D - 43{\cdot}2°$ (in ethyl alcohol (96%)). Colour reactions: Salkowski's test; acid yellow-red with green fluorescence; chloroform uncoloured. Liebermann-Burchardt test: chloroform blue then green; acid violet.

SUMMARY.

1. The carotenoids of the furze (*Ulex europaeus*) and of Planchon's furze (*Ulex galli*) have been investigated. α-Carotene, β-carotene, violaxanthin, taraxanthin and an isomeride of lutein were isolated in pure crystalline state.

2. The occurrence is proved of an unknown carotenoid with an absorption spectrum similar to that of flavoxanthin, but with other chemical properties. The occurrence of flavoxanthin in *U. galli* is probable.

3. Hentriacontane, a sterol of the formula $C_{30}H_{50}O$ and sitosterol were isolated.

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CCLXXV. STUDIES ON CAROTENOIDS.[1]

IV. THE CAROTENOIDS OF *GENISTA TRIDENTATA*.

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THE systematic investigation of the metabolic products of related plant species is of considerable biochemical interest, as it can provide us with a view of similar vital processes which occur during the metabolism and assimilation of the plants. Therefore we believe it useful to include in our studies of the carotenoids of the Portuguese flora a systematic investigation of the family of Leguminosae for later comparison of the results obtained.

In the preceding paper we have communicated the results of the investigation of two species of furze, and we propose to describe here our results obtained with the flowers of *Genista tridentata*.

The flowers of two other species, i.e. *G. racemosa* and *G. tinctoria*, have already been examined by Tammes [1900] and Courchet [1888] who determined the presence of carotenoids in these plants. Recent investigations of Baker & Robinson [1925; 1926; 1928] showed the presence in *G. tinctoria* of two water-soluble colouring matters, genistein, $C_{15}H_{10}O_5$ (3:7:4′-trihydroxy*iso*flavone), and luteolin, $C_{15}H_{10}O_6$ (5:7:3′:4′-tetrahydroxyflavone).

METHODS.

3·7 kg. of dried yellow flowers were ground in a mill to a very fine powder and this was then extracted several times with a total quantity of 30 litres of light petroleum. The extract was concentrated *in vacuo* in a current of carbon dioxide to 150 ml., and to the concentrate 450 ml. of absolute ethyl alcohol were added. On standing in the ice-box for several days a large amount of colourless material separated. This was removed by filtration, and water was added to the filtrate. After prolonged washing with water, the solution was dried over anhydrous sodium sulphate, filtered through a dry filter-paper and diluted to 600 ml. This solution was used for chromatographic analysis.

The colouring matter was adsorbed on to a column of activated aluminium oxide, 18 cm. long and 6 cm. in diameter. On washing the column with light petroleum nine different zones developed, of which, however, only seven were used for further treatment, the two upper zones being oxidation products.

Zone

1. Dark green, narrow and sharp
2. Yellow, narrow and sharp
3. Green, broad and diffuse
4. Light yellow, broad and diffuse
5. Orange, very broad and sharp
6. Orange-red, broad and sharp
7. Orange-red, narrow and diffuse

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[1] Aided by a grant from the van't Hoff Foundation, Amsterdam.

The column was divided into three Parts, zones 1–4 forming Part I, zone 5 which occupies most of the column forming Part II and zones 6 and 7 forming Part III.

Part I. The colouring matter was eluted with light petroleum containing 1 % of methyl alcohol and the solution, which contains the chlorophyll pigments and a part of the esterified xanthophyll, was concentrated to 100 ml. *in vacuo.* To the concentrate were added 50 ml. of concentrated methyl alcoholic KOH and a quantity of absolute ethyl alcohol sufficient to make the mixture homogeneous. After complete saponification 500 ml. of light petroleum were added and water to reduce the alcohol concentration to about 40 %. The alcoholic layer was twice extracted with a total of 500 ml. of light petroleum, the united solutions of light petroleum were washed several times with water and then dried over anhydrous sodium sulphate. This solution served for a further chromatographic analysis.

The solution was adsorbed on to activated calcium carbonate and the chromatogram developed by washing with a mixture of light petroleum-benzene 4:1. Four distinct zones developed:

	Absorption bands in light petroleum (B.P. 80°) mμ
1. Narrow yellow zone	467, 441
2. Broad pale yellow zone	474, 441
3. Broad yellow-red zone	475 (very faint)
4. Broad orange-yellow zone	Very faint bands

From the first zone we have isolated a sterol which we shall describe later. It was, however, not possible to crystallize the colouring matter contained in this zone, which gives a strong blue colour with concentrated hydrochloric acid in ethereal solution.

The second and third zones, which have the same absorption spectra, were united and eluted with methyl alcohol. The solution was concentrated *in vacuo* to 10 ml. and kept in the ice-box for 1 day. A colourless substance crystallized, which was removed by suction. The filtrate was then evaporated to dryness *in vacuo* and the residue dissolved in light petroleum and kept in the ice-box. A crystalline precipitate appeared which was removed by filtration in the cold, as it was soluble at ordinary temperature. The xanthophyll was then extracted from the light petroleum solution with methyl alcohol (90 %), the solution concentrated *in vacuo* to 5 ml. and kept in the ice-box for 2 days. A mixture of xanthophyll with colourless substances crystallized. The crystals were filtered off, washed with light petroleum and twice recrystallized from absolute methyl alcohol. Pure lutein was obtained; M.P. 191–192° (uncorr., in evacuated tube). Absorption bands in methyl alcohol 471, 445 mμ; in CS_2 504, 472 mμ.

The fourth zone of the chromatogram which contained only a very small portion of colouring matter was not further investigated.

Part II. The colouring matter of this part of the chromatogram was eluted with light petroleum containing 1 % of methyl alcohol and the solution concentrated to a small volume *in vacuo.* 70 ml. of concentrated methyl alcoholic KOH and enough absolute ethyl alcohol to make the solution homogeneous were added. After complete saponification the solution was filtered from a colourless precipitate and a large quantity of water added. The formation of an emulsion took place, and by saturating this with sodium chloride the colouring matter was precipitated. It was dissolved in methyl alcohol and the solution reduced to a small volume *in vacuo.* A xanthophyll crystallized together with colourless substances. The precipitate was dissolved in a small volume of methyl alcohol and three volumes of ether were added which caused the precipitation of a colourless water-soluble substance. After removing this by centrifuging the solution was evaporated to dryness *in vacuo* and the residue dissolved in methyl alcohol. This solution was again evaporated to dryness and the residue dissolved in hot light petroleum. On keeping the solution in the ice-box for 24 hours, a xanthophyll crystallized which after two further crystallizations from absolute methyl alcohol proved to be pure lutein; M.P. 192° (uncorr., in evacuated tube); absorption bands in methyl alcohol 473, 446 $m\mu$; in CS_2 509, 474 $m\mu$.

Part III. After elution of the colouring matter with a mixture of light petroleum and methyl alcohol, the solution obtained was concentrated *in vacuo* to 300 ml. To the concentrate 50 ml. of a concentrated methyl alcoholic solution of KOH, 50 ml. of ethyl alcohol (96 %) and 100 ml. of absolute ethyl alcohol were added and the mixture was saponified for 2 days with occasional shaking. Part of the colouring matter passed into the alcoholic layer. After separation of the two layers, the light petroleum solution was twice washed with methyl alcohol (90 %) and the alcoholic layers were united. Water was added to lower the concentration of the alcohol to 80 % and the solution then extracted with light petroleum. In this way two solutions were obtained, an alcoholic solution containing xanthophyll and a light petroleum solution containing hydrocarbons.

(*a*) *Xanthophyll.* To the alcoholic solution a little light petroleum and then a considerable quantity of water were added with continuous shaking. As an emulsion formed, the solution was saturated with sodium chloride which caused the precipitation of 650 mg. of xanthophyll in a crystalline state. After two recrystallizations from absolute methyl alcohol we obtained 204 mg. of very pure lutein with M.P. 193° (uncorr., in evacuated tube) and absorption bands in CS_2 507, 473 $m\mu$. From the mother-liquors more lutein of a less degree of purity was obtained by concentration.

(*b*) *Hydrocarbons.* The light petroleum solutions were thrice washed with methyl alcohol (90 %) and then several times with water, dried over anhydrous sodium sulphate and then passed through a dry filter. The solution was then adsorbed on to activated aluminium oxide and the column washed with light petroleum. Three different zones formed:

	Absorption bands in light petroleum (B.P. 80°) $m\mu$
1. Very narrow red-violet zone	453, 424
2. Broad sharp orange zone	483, 448
3. Narrow diffuse yellow zone	476, 446

The first zone contained a very small amount of a carotenoid which shows similar absorption bands to those of flavoxanthin and which is identical with a carotenoid found in the furze, described in the preceding paper. It could not be obtained in a pure crystalline state.

The second zone contains the bulk of the colouring matter and consists of β-carotene. It was eluted with light petroleum containing 1 % of methyl alcohol. This solution after colorimetric determination [Kuhn & Brockmann, 1932] was found to contain 191 mg. of carotene. It was concentrated *in vacuo* to 100 ml. and 50 ml. of absolute ethyl alcohol were added. After standing in the ice-box for 12 hours, 76 mg. of pure β-carotene had separated in beautiful crystals of permanganate-like colour; M.P. 181–182° (uncorr., in evacuated tube); absorption bands in CS_2 521, 487 $m\mu$.

The third zone was also eluted with the mixture of light petroleum-methyl alcohol and evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml. of light petroleum and 30 ml. of absolute methyl alcohol were added. As the colouring matter did not crystallize, the solution was brought to dryness and the residue dissolved in 10 ml. of hot methyl alcohol. From this solution about 1 mg. of α-carotene crystallized, which, however, was not pure. Owing to the small quantity, we have not purified this substance further.

Sitosterol. This sterol was isolated as a by-product from zones 2 and 3 of the chromatogram of Part 1. It crystallizes from methyl alcohol in characteristic leaflets with strong birefringency; M.P. 131° (uncorr.); $[\alpha]_D$ −34·1° (in ethyl acetate). With the reagents of Salkowski and of Liebermann-Burchardt the sterol gives the colour reactions characteristic of sitosterol. We obtained 177 mg. of the pure product.

In addition to sitosterol another sterol was isolated in very small amount from zone 1 of the same chromatogram, which gives the same colour reactions as the sterol $C_{30}H_{50}O$ described in the preceding paper. The low M.P. of 105–106° (uncorr.) indicated that it was probably not pure, but owing to the small amount of crystals we could not purify it further.

DISCUSSION.

There is a striking difference between the carotenoids of the furze and those of *G. tridentata* in respect of their xanthophyll contents. Whilst in the first the xanthophylls are a mixture in which violaxanthin predominates, with taraxanthin and only a little lutein, other xanthophylls being probably present, the xanthophyll of the latter is almost pure lutein. The lutein occurs in the plant in an esterified form and, by means of chromatographic analysis, we were able to demonstrate that there exist at least three different esters of lutein.

On the other hand, the carotene of *G. tridentata* is almost pure β-carotene, only traces of the α-isomeride being present. From this it is clear that the hydrocarbons belong to the β-carotene series, whereas xanthophyll in its chemical structure is related to α-carotene. A similar relation exists in a less accentuated degree between the carotene and xanthophyll of the green leaves of almost all plants. It is possible to admit that *in vivo* lutein may be related to β-carotene, in contrast to the relation *in vitro*.

SUMMARY.

1. The carotenoids of *Genista tridentata* have been investigated. α-Carotene, β-carotene and lutein have been isolated in a crystalline state. The occurrence of other carotenoids is probable.
2. Sitosterol and another sterol were isolated.
3. A possible biological relationship between β-carotene and lutein is discussed.

The authors wish to express their thanks to the Director of the Institute, Prof. Dr A. de Morais-Sarmento, for his great interest during the course of the work and for his many valuable suggestions. Acknowledgement is also made to Prof. Dr West of the English Institute of the University for revising the manuscript.

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CCLXXVI. THE ACTIVATION OF THE MALE SEX HORMONES. I.

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(*Received 20 August 1936.*)

1. *The activation of testosterone by the addition of organic acids.*

It is well known that in the rat test testosterone exhibits a greater activity than androsterone, especially on the seminal vesicles of castrated rats. As we have recently shown [1936, 1, 2] this only applies to oily solutions of testosterone; testosterone is, strangely enough, hardly active when dissolved in paraffin oil or in aqueous solutions of glycerol. We found, however, that after the addition of certain carboxylic acids, especially of the fatty acid series such as ricinoleic acid, the activity reappeared. The effect of solutions in fatty oils, like sesame oil, is increased to a considerable degree by such saturated and unsaturated fatty acids.

Meanwhile the number of the acids investigated has been considerably augmented and the earlier particulars supplemented. The weights of the seminal vesicles, prostate, Cowper's glands and preputial glands attained after administration of testosterone in combination with the new acids tested and with those of which the examination has been extended are given in Table I.

Technique. The rats weighed 60–80 g. at the time of castration; the experiments were not made until 25–30 days after castration. The animals received once a day a dose of 50γ testosterone during 10 days, subcutaneously injected together with the quantity of acid mentioned dissolved in 0·5 ml. sesame oil. The rats were killed on the 11th day and the accessory organs were weighed immediately. Fatty acids which are solid at room temperature, e.g. hydroxy-acids and stearic acid, frequently had to be dissolved and injected at body temperature on account of their low solubility.

The acids are arranged in the table approximately according to decreasing activity. Some of them, e.g. suberic, mandelic, tropic, nicotinic, piperic and α-naphthoic acids, are only very slightly soluble and for this reason their solutions were almost inactive; thus the question whether the acids themselves are active or not was left open. Some proved to be toxic or produced pronounced local irritation, e.g. glycerophosphoric, undecylenic, phenylpropiolic, tetrolic, phenylacetic, lactic, pyruvic, acetic and formic acids, with which therefore the values obtained varied considerably. The irritating effect of the latter acids is probably due to their high acidity. Saturated higher fatty acids such as palmitic acid produce practically no irritation.

As will easily be seen from Fig. 1 the increase of activity can attain a considerable value with increasing doses of the acid. Still higher doses generally caused technical difficulties due to the saturation limit being attained or to

Table I.

Acid used + 50 γ testosterone daily in 0·5 ml. sesame oil	Daily quantity of acid in mg.	Weight of the glands in mg.				No. of rats per experiment
		Seminal vesicles	Prostate	Cowper's glands	Preputial glands	
ω-Hydroxytridecanoic acid, $C_{13}H_{26}O_3$	10	132	124	154	108	2
ω-Hydroxymargaric acid, $C_{17}H_{34}O_3$	20	184	194	155	98	2
ω-Hydroxypalmitic acid, $C_{16}H_{32}O_3$	25	150	158	175	91	2
Pyruvic acid, $C_3H_4O_3$	10	85	90	134	80	2
Glycerophosphoric acid, $C_3H_9O_6P$	10	80	110	120	92	2
Palmitic acid, $C_{16}H_{32}O_2$	50	235	210	205	91	6
	25	115	150	170	80	4
	10	52	101	130	64	4
	5	40	75	88	41	4
λ-Hydroxystearic acid, $C_{18}H_{36}O_3$	50	210	220	192	95	2
	25	170	190	175	97	4
	10	70	103	105	48	4
Ricino-elaidic acid, $C_{18}H_{34}O_3$	10	78	105	91	51	4
	5	47	88	88	52	2
ω-Hydroxypentadecanoic acid, $C_{15}H_{30}O_3$	25	140	159	168	108	2
ω-Hydroxypelargonic acid, $C_9H_{18}O_3$	10	70	114	107	58	2
Behenic acid, $C_{22}H_{44}O_2$	50	165	202	228	86	8
	25	141	160	156	78	4
Camphor-3-carboxylic acid, $C_{11}H_{16}O_3$	25	106	124	128	78	6
λ-Ketostearic acid, $C_{18}H_{34}O_3$	25	95	125	130	75	4
ω-Hydroxydecanoic acid, $C_{10}H_{20}O_3$	25	84	122	138	80	2
Elaidic acid, $C_{18}H_{34}O_2$	25	62	100	112	48	2
	5	34	58	78	55	2
Acetic acid, $C_2H_4O_2$	10	65	70	—	—	2
Lactic acid, $C_3H_6O_3$	10	60	80	78	66	4
Arachidic acid, $C_{20}H_{40}O_2$	50	135	182	174	96	4
	25	117	168	170	83	3
β-Phenylpropionic acid, $C_9H_{10}O_2$	25	75	78	86	—	2
Propionic acid, $C_3H_6O_2$	50	117	158	150	70	3
	25	74	131	—	72	2
Hexahydrobenzoic acid, $C_7H_{12}O_2$	25	70	75	80	—	2
Benzoic acid, $C_7H_6O_2$	50	102	123	128	76	4
n-Hexanoic acid, $C_6H_{12}O_2$	50	95	140	130	71	4
Undecylenic acid, $C_{11}H_{20}O_2$	25	65	95	112	54	2
Sulphoricinoleic acid, $C_{18}H_{34}O_6S$	50	80	121	117	73	2
Pelargonic acid, $C_9H_{18}O_2$	25	55	98	107	—	2
Cinnamic acid, $C_9H_8O_2$	50	60	75	77	58	2
ω-Hydroxyundecanoic acid, $C_{11}H_{22}O_3$	25	56	90	98	65	2
Phenylacetic acid, $C_8H_8O_2$	25	52	85	—	—	2
Phenylpropiolic acid, $C_9H_6O_2$	25	46	88	95	41	2
Tetrolic acid, $C_4H_4O_2$	25	30	64	—	—	2
Testosterone alone	—	42	66	75	55	4
Sesame oil	—	14	41	49	57	3

their irritating effect. The relationship between constitution and effect of fatty acids as described in the earlier papers [1936, 1, 2] can be summarized as follows:

Saturated fatty acids are more effective than unsaturated fatty acids.

Simply unsaturated fatty acids are more effective than unsaturated fatty acids with several double bonds.

Unsaturated fatty acids of the *trans*-series are more effective than unsaturated fatty acids of the *cis*-series.

Unsaturated hydroxylated fatty acids are more effective than unsaturated fatty acids without a hydroxyl group

From this we deduced that the hydroxylated saturated fatty acids should prove to have the greatest activity. This is confirmed, as will be seen from Fig. 2 in which the series of saturated fatty acids is compared with a number of saturated hydroxy-fatty acids[1] in respect of the produced weight of the seminal vesicles.

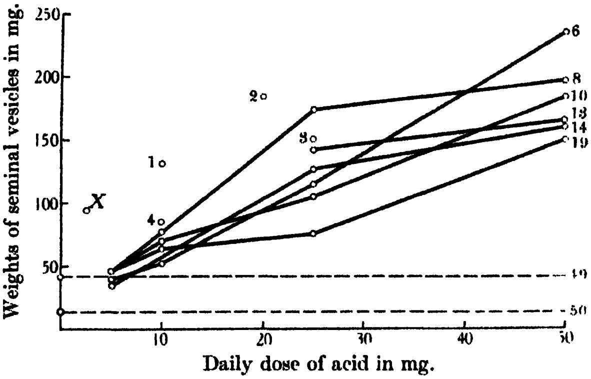

Fig. 1. Effect of the addition of some organic acids to testosterone on the weight of seminal vesicles. 1, ω-Hydroxytridecanoic acid; 2, ω-hydroxymargaric acid; 3, ω-hydroxypalmitic acid; 4, pyruvic acid; 6, palmitic acid; 8, ricino-elaidic acid; 10, stearic acid; 13, behenic acid; 14, behenolic acid; 19, brassidic acid; 49, testosterone alone; 50, sesame oil.

X, "natural" *X*-substance preparation from urine.

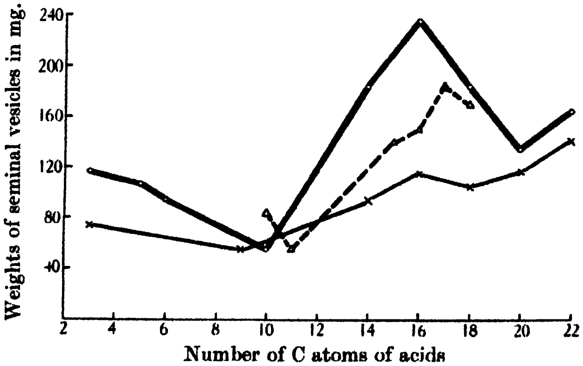

Fig. 2. Effect of hydroxylated and non-hydroxylated fatty acids on the activity of testosterone in relation to the number of C atoms of the acids.

━━ Daily 50 mg. saturated normal fatty acid
× —— × Daily 25 mg. saturated normal fatty acid
△ - - - △ Daily 25 mg. saturated normal hydroxylated fatty acid
▲ Only 20 mg. acid
} + 50γ testosterone.

The dependence of the effect of the fatty acids upon the number of their carbon atoms is striking. Near C_{10} a minimum action is attained whilst near C_{16} a pronounced maximum is observed. Whether with higher acids than C_{22} a still further increase in activity would result is problematical and requires further investigation.

A marked effect is observed with lactic acid and especially with pyruvic acid, an α-keto-acid, in contrast to λ-ketostearic acid. It may be worth while

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[1] The ω-hydroxy-fatty acids were kindly supplied to us by Messrs Firmenich and Co., Geneva, through the courtesy of Prof. Ruzicka.

Table II.

	Daily dose γ	Without palmitic acid Weight of the glands in mg.					With 50 mg. palmitic acid daily Weight of the glands in mg.				
		Seminal vesicles	Prostate	Cowper's glands	Preputial glands	No. of rats	Seminal vesicles	Prostate	Cowper's glands	Preputial glands	No. of rats
3-*cis*-Androsterone	2000	142	260	206	142	2	149	270	180	130	2
	500	68	131	120	107	2	51	158	90	70	2
3-*trans*-Androsterone	1000	14	39	40	45	2	14	38	52	55	2
17-*trans*-Testosterone (testis hormone)	500	127	173	165	88	2	354	350	241	150	2
	200	124	176	162	81	4	272	280	212	110	4
	100	62	86	140	58	4	263	263	206	90	4
	50	40	70	74	55	4	220	209	165	78	4
	25	—	—	—	—	—	117	200	120	61	4
	10	—	—	—	—	—	37	76	97	60	4
	5	—	—	—	—	—	32	75	88	40	4
17-*cis*-Testosterone	1000	15	41	50	80	3	15	43	60	85	3
17-Methyltestosterone	200	140	167	156	94	2	—	—	—	—	—
	100	80	98	130	80	3	—	—	—	—	—
	50	58	88	75	51	3	160*	210*	162*	88*	3*
17-Ethyltestosterone	500	107	129	161	82	2	—	—	—	—	—
	200	44	57	70	51	2	74	86	105	60	2
17-Ethyldihydrotestosterone	500	49	108	113	61	2	70	148	157	78	2
	200	19	55	60	48	2	40	60	107	80	2
	100	13	45	46	46	—	—	—	—	—	—
	50	14	36	44	30	—	—	—	—	—	—
Androstane-3-*cis*-17-*trans*-diol	200	74	140	145	89	4	239	308	210	161	4
	100	43	85	123	68	4	—	—	—	—	—
Androstene-3-*trans*-17-*trans*-diol	1000	48	71	80	83	2	114	136	156	153	2
	500	30	50	81	107	2	99	120	152	177	2
Androstanedione	500	30	108	89	73	4	28	122	120	83	2
Androstenedione	500	126	166	176	101	4	208	251	196	130	3
	200	90	130	150	80	4	—	—	—	—	—
Sesame oil alone	—	14	41	49	57	3	14*	38*	45*	40*	2*

* Values obtained with brassidic acid instead of palmitic acid.

to study still higher α-hydroxy- or α-keto-fatty acids. The apparently very high activity of ω-hydroxytridecanoic acid requires still further investigation; in fact the number of experiments made with most of the acids was insufficient to yield definite results. Glycerophosphoric acid showed very high activity.

II. *The activation of other compounds with male hormone activity by the addition of organic acids.*

Table II shows the effect of palmitic acid (or brassidic acid) on a number of other male hormone preparations. The results obtained with testosterone are also given for comparison. From this it is seen that the activities of *cis*- and *trans*-androsterone, *cis*-testosterone and androstanedione are not changed; a moderate effect is observable in the case of the 17-ethyl compounds (17-ethyltestosterone and 17-ethyldihydrotestosterone) and a still greater effect in the case of androstenedione and *trans*-androstenediol. The most pronounced influence, however, is exerted in the cases of androstane-3-*cis*-17-*trans*-diol, 17-methyltestosterone and 17-*trans*-testosterone. It certainly seems that a hydroxyl group in the 17-*trans*-position in combination with one in the 3-*cis*-position or with an α, β-unsaturated keto-group in the 3-position contributes specially to the activating effect.

Deanesly & Parkes [1936] recently confirmed our findings as regards the possibility of increasing the effect of an oily solution of testosterone by addition of palmitic acid. Contrary to us they found that the effect of androsterone, given in a total dose of 10 mg., could also be increased to a certain degree.

III. *The activation of testosterone by the addition of alcohols etc.*

Since the hydroxy-acids exhibited marked activating properties it was interesting to study the effect of the monohydric alcohols. As seen from Table III the activating power of these alcohols is very considerable[1] and attains a

Table III.

Preparation used + 50 γ testosterone daily in 0·5 ml. sesame oil	Daily quantity of alcohol mg.	Weight of the glands in mg.				No. of rats per experiment
		Seminal vesicles	Prostate	Cowper's glands	Preputial glands	
Ethyl alcohol, C_2	50	82	98	106	61	2
Propyl alcohol, C_3	50	117	156	152	66	2
*iso*Propyl alcohol, C_3	50	120	150	145	80	2
Butyl alcohol, C_4	50	72	110	130	68	4
Hexyl alcohol, C_6	50	95	125	120	70	4
Octyl alcohol, C_8	50	77	115	125	50	2
Dodecyl alcohol, C_{12}	50	95	98	120	72	2
Cetyl alcohol, C_{16}	50	125	165	185	95	4
Stearyl alcohol, C_{18}	50	200	220	210	71	4
Oleyl alcohol, C_{18}	50	87	111	110	59	2
Cinnamyl alcohol, C_9	50	80	81	70	45	2
Ethyl propionate	50	33	66	70	48	2
Methyl butyrate	50	44	65	80	48	2
Methyl valerate	50	56	80	78	55	2
Methyl stearate	50	43	89	75	54	2
Monostearin	50	40	76	72	56	4
Testosterone alone	—	42	66	75	53	—
Sesame oil	—	14	41	49	57	—

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[1] It was established by titration that the activity of these alcohols is not due to admixture with the corresponding fatty acids.

strikingly high maximum with stearyl alcohol. (These values were obtained under the same experimental conditions as those used in previous experiments.) On the other hand, the corresponding unsaturated oleyl alcohol is much less effective. As already mentioned, testosterone in glycerol—a trihydric alcohol—is completely ineffective. It is still doubtful whether testosterone is also inactive in entirely pure triglycerides since one cannot exclude the possibility of the oils previously tested [Miescher *et al.* 1936, 2] containing some "natural" X-substances.

Table III likewise gives information as to the addition of fatty acid esters to oily solutions of testosterone; methyl valerate alone shows a slight effect.

It was natural to suspect a certain connexion between activation and surface activity. In fact it has been found that some of the known wetting agents are likewise more or less effective, but very irritating.

IV. *The activation of testosterone by the addition of "X-substances".*

David *et al.* [1935] described for the first time the activating action of certain acid fractions of extracts derived from testes and other organs, or from body fluids like urine, in combination with testosterone. From Fig. 1 it will be observed that 3 mg. of an X-substance preparation, obtained by Dr Locher in our laboratories from urine, together with 50γ testosterone will increase the weight of the seminal vesicles from 14 to 95 mg., that is to say about treble the increase produced by testosterone alone. This effect is approximated only by that produced by some hydroxy- and keto-acids.

The Amsterdam investigators [Polak *et al.* 1936, 1, 2] believe that there exists a specific difference between the "natural" X-substance[1] and the carboxylic acids found effective by us. However, this appears to us hardly probable. As Deanesly & Parkes have already reported, the difference is more quantitative than qualitative. Some time ago [1936, 1, 2] we drew attention to the fact that large quantities of free palmitic acid are present in the testes. Besides this, however, there exists in them an apparently very complex mixture of other, not yet identified, but also effective acids. It is quite probable that all the acids present participate in the general activating action according to the quantity in which they are present and the degree of their activity. Possibly the attainment of an optimum effect depends on the proportion in which the acids are mixed. If we understand correctly the investigators of the Amsterdam group, the difference between our carboxylic acids and the X-substances lies in the fact that the latter not only improve the absorption of the hormones at the site of injection but also enhance the effect of the hormones on the test organs. This, however, has not been proved. It is also doubtful if any acids, e.g. those found in the testes, or even those found in the urine, are connected with the activation of the testicular hormone under natural conditions [see also Miescher *et al.* 1936, 3].

The assumption of Polak *et al.* [1936, 1, 2] that all our effective acids could contain small amounts of the natural X-substance does not seem probable since our list also includes synthetically prepared acids of high activity.

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[1] These authors specially point out that one of their X-substance preparations isolated from urine is effective in a daily dose of 0·3 mg. together with 70γ androstanediol. The weights of the seminal vesicles and of the prostata amounted to only 25 mg. each in comparison with 14 and 25 mg. respectively with androstanediol alone; therefore it is difficult to compare their findings with ours. We preferred for our experiments greater differences which are far beyond the limits of error.

Finally it may be mentioned that the X-substance, as Freud *et al.* [1935] have shown, activates testosterone and androstanediol, but not androsterone. This fact agrees with our findings regarding the use of palmitic acid and points likewise to an effect of similar nature.

SUMMARY.

1. The activating effects of about 40 carboxylic acids on the effect of testosterone have been tested on castrated rats. These experiments supplemented earlier tests. It was observed that the saturated normal fatty acids exhibit certain regularities. In this series, acids with about 10 carbon atoms showed a minimum action while those with about 16 carbon atoms showed a decided maximum. Hydroxylated saturated or unsaturated fatty acids are as a rule more effective than saturated or unsaturated fatty acids without a hydroxyl group.

2. *cis*- and *trans*-Androsterone as well as androstanedione are not activated by palmitic acid. The most pronounced effect is observed with those male hormone preparations which, like testosterone, methyltestosterone and androstane-3-*cis*-17-*trans*-diol, possess a hydroxyl group in the 17-*trans*-position in combination with a hydroxyl group or with an α, β-unsaturated keto-group in the 3-position.

3. In the rat test monohydric alcohols also increase the effect of testosterone. Of those tested, the saturated stearyl alcohol is the most effective whilst the unsaturated oleyl alcohol is less effective. This is in agreement with our findings in the series of fatty acids where also the saturated are more active than the unsaturated. Certain wetting agents also exhibit an activating effect.

4. Acid fractions obtained from testes contain, in addition to palmitic acid, other more effective components. Probably the so-called natural activator represents a mixture of acids which differ only quantitatively as regards their activating effects. The question is left open to what extent, if any, such acids actually influence the activity of the genuine male hormones of the testes.

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CCLXXVII. THE ACTIVATION OF THE MALE SEX HORMONES. II.

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(*Received 20 August 1936.*)

I. *The activation of testosterone by esterification.*

We showed in an earlier paper [1936, 1] that on injecting testosterone and palmitic acid at different sites in the body of the rat an activating effect could no longer be observed when the sites of injection were too far apart. In our opinion this suggested that the carboxylic acids have no effect on the sex organs themselves, but rather a local effect at the site of injection, in creating more favourable conditions for the absorption of the administered solution. Deanesly & Parkes [1936, 1], who confirmed the fact itself, suggested that the increased effect was due to retardation of the absorption of testosterone. They point to the retarded effects of benzoates of oestrone and oestradiol and are of the opinion that one cannot exclude the possibility of esterification of testosterone by the acid added, even during the preparation of the solution. However, the possibility of activating testosterone by primary alcohols and especially the fact that we found testosterone palmitate and stearate to have practically no effect are in conflict with this theory.

We decided, however, to investigate thoroughly the problem of testosterone esters. Testosterone acetate and benzoate had already been tested. Tschopp [1936] found the former to be just as effective as testosterone, whilst according to Deanesly & Parkes [1936, 2] the latter is practically ineffective on the capon's comb. In order to compare these findings we prepared a new series of testosterone esters with aliphatic acids.[1] Information is given in Table I concerning the properties of the two already known and the nine new esters.

Table I.

	Number of C atoms of the acid residue	M.P. of the ester (corr.)	One international (capon's comb) unit is equivalent to γ	Number of international units contained in 1 mg.
Formate	1	127–129°	20	50
Acetate	2	140–142°	20	50
Propionate	3	121–123°	20	50
n-Butyrate	4	111–113°	60	17
*iso*Butyrate	4	134–136°	70	14
n-Valerate	5	109–111°	200	5
*iso*Valerate	5	138–140°	250	4
n-Decanoate	10	55–57°	350	3
Palmitate	16	72–74°	>1000	<1
Stearate	18	79–80°	>1000	<1
Benzoate	7	198–200°	>1000	<1

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[1] See also our preliminary report [1936, 2]. The chemical part of this work will be published by Ruzicka & Wettstein [1936].

(*a*) *Effect of the esters on the capon's comb. Technique.* The daily dose of the compounds investigated was dissolved in 0·5 ml. sesame oil and injected for 6 days into healthy capons. The increase and decrease of the surface of the comb was controlled by measuring the shadow pictures by a planimeter.

The effects of the various esters are to be seen on Fig. 1. The graphs show the average values obtained on 3–4 animals. It will be seen that the actions of the formate, acetate and propionate are the most rapid and pronounced. The duration of the effect is somewhat longer with the propionate. The intensity of action then decreases rapidly as the number of carbon atoms of the esterified acids increases. It disappears almost entirely with palmitate, stearate and benzoate.

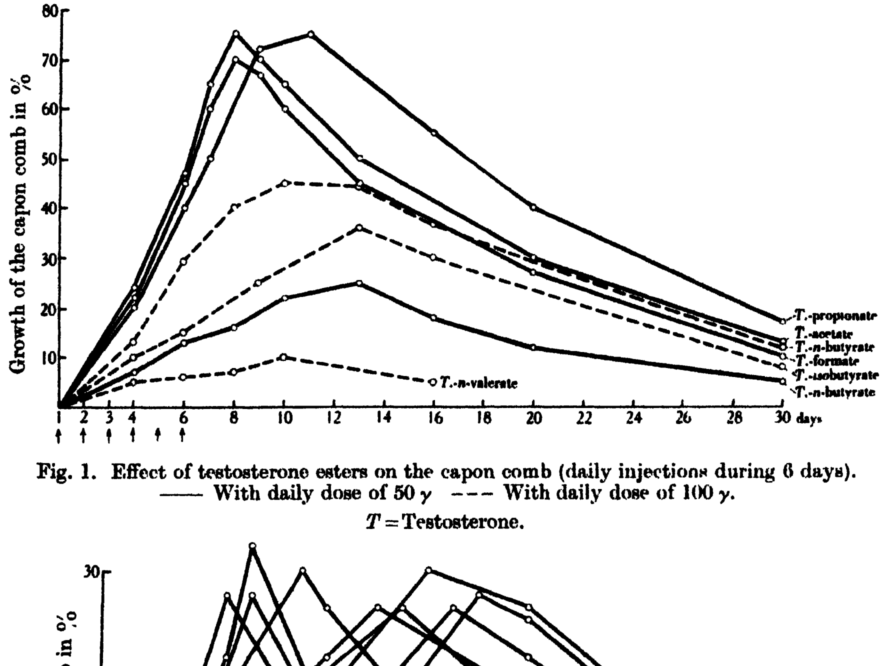

Fig. 1. Effect of testosterone esters on the capon comb (daily injections during 6 days). —— With daily dose of 50 γ --- With daily dose of 100 γ.
T = Testosterone.

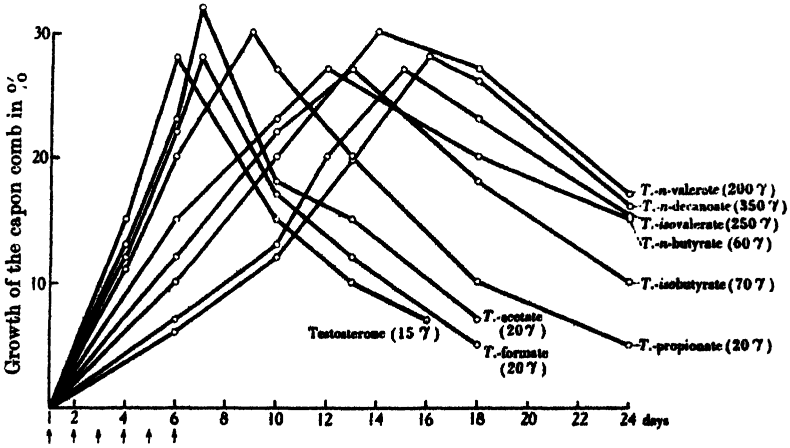

Fig. 2. Time course of effect of approximately 1 international capon unit of different testosterone esters on the capon comb.
T = Testosterone.

To convert the results into international units the doses of the esters were reduced to such an extent that a maximum increase of the surface of the comb amounting to about 30% of the original surface was obtained, equal to the action of 100γ androsterone under our test conditions.[1] From Fig. 2 it will be seen distinctly that the higher the mol. wt. of the esterified acid, the later the maximum increase of the surface of the comb is attained. For comparison it should be said that, conditions being equal, one international unit, i.e. 100γ androsterone, corresponds to the activity of 15γ testosterone. (Deanesly and Parkes found previously one unit in 17γ testosterone and Tschopp [1936], under somewhat different conditions, 1 unit in 13γ.)

The addition of palmitic acid does not effect either the activity of testosterone or its esters on the capon's comb.

(*b*) *The effect of esters on castrated rats.* The esters were also thoroughly tested on the castrated rat. All the animals used in the experiments were castrated at a weight of 60–80 g. and the tests not begun until 16–21 days after castration.[2] We employed three methods.

First method using ten injections. The castrated animals received the indicated doses, dissolved in 0·5 ml. sesame oil, by subcutaneous injection daily during 10 days. On the 11th day the animals were killed and the sex organs weighed. Table II gives the results and Fig. 3 demonstrates graphically the effect especially on the seminal vesicles. The surprising fact emerges that the lower esters[3] are far more effective than testosterone. The effects of some of these esters exceed even those produced by testosterone plus 50 mg. palmitic acid. On

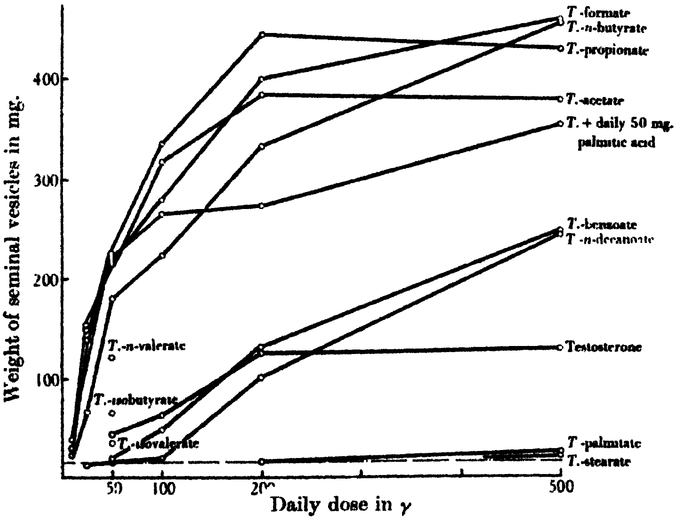

Fig. 3. Effect of testosterone esters on the weight of seminal vesicles (method using ten injections).
T = Testosterone.

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[1] We observed in all cases the same maximum increase independent of the day on which it appeared; thus a simultaneous comparison of the effects was not made.

[2] However, in all the experiments using acids as activators this interval was 25–30 days.

[3] In a private communication Dr Girard of Paris informs us that he also saw more favourable effects on the general condition of old capons following administration of testosterone acetate in high doses.

Table II.

	Daily dose γ	Weight of the glands in mg.				Number of rats
		Seminal vesicles	Prostate	Cowper's glands	Preputial glands	
Testosterone formate	500	460	360	240	146	2
	200	400	363	283	136	4
	100	281	290	217	111	6
	50	214	225	215	96	4
	25	153	171	171	70	4
	10	37	60	90	55	2
	5	32	64	88	36	2
Testosterone acetate	500	380	356	220	137	4
	200	383	357	228	136	2
	100	317	298	227	104	5
	50	218	241	195	86	8
	25	142	180	175	75	4
	10	30	62	83	49	2
Testosterone propionate	500	430	380	266	145	2
	200	444	375	244	131	4
	100	334	299	190	101	4
	50	225	270	190	89	4
	25	150	155	155	65	4
	10	30	50	50	36	2
	5	20	47	62	33	2
Testosterone *n*-butyrate	1000	466	422	258	202	2
	500	458	395	255	155	4
	200	332	383	233	104	4
	100	223	290	231	86	4
	50	181	185	190	73	4
	25	64	102	152	70	4
	10	22	58	81	45	2
	5	16	48	59	55	2
Testosterone *iso*butyrate	50	64	94	105	60	4
Testosterone *n*-valerate	50	122	152	146	60	4
Testosterone *iso*valerate	50	34	55	77	50	4
Testosterone *n*-decanoate	500	246	185	183	92	2
	200	100	95	116	61	3
	100	19	47	58	35	4
	50	15	38	50	45	4
	25	12	34	43	35	4
	10	13	35	40	30	2
	5	11	28	42	28	2
Testosterone benzoate	500	247	254	205	103	2
	200	130	142	136	80	4
	100	47	64	84	46	4
	50	17	42	50	39	8
Testosterone stearate	1000	30	50	50	32	2
	500	19	39	45	37	2
Testosterone palmitate	500	22	45	47	36	2
	200	17	43	45	37	2
Testosterone	500	127	173	165	88	2
	200	124	176	162	81	4
	100	62	86	140	58	4
	50	42	66	75	55	4
Testosterone + daily 50 mg. palmitic acid	500	354	350	241	150	2
	200	272	280	212	110	4
	100	263	263	206	90	4
	50	220	209	165	78	4

the other hand palmitic acid does not change the effect of, for example, testosterone acetate (see section III). Whilst the formate, acetate, propionate and butyrate show practically the same effect, the latter decreases rapidly when we pass to higher esters and to those containing a branched instead of a straight chain acid residue. Palmitate and stearate are almost ineffective, whilst benzoate and *n*-decanoate in higher doses show pronounced activities in this test.

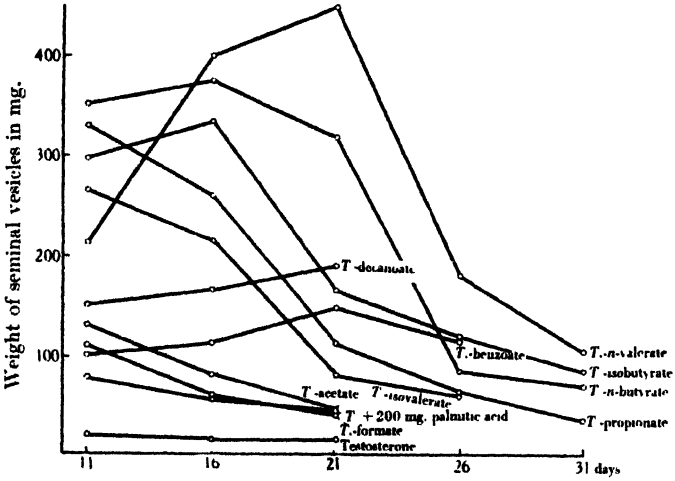

Fig. 4. Effect of testosterone esters on the weight of seminal vesicles (method using two injections on the 1st and 6th day).
T = Testosterone.

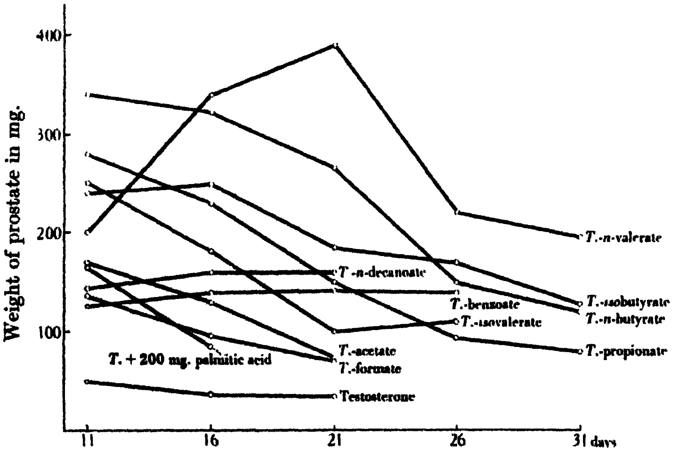

Fig. 5. Effect of testosterone esters on the weight of prostate (method using two injections on the 1st and 6th day).
T = Testosterone.

It is worth mentioning that the maximum effect is obtained already with small doses of the lower esters; higher doses do not materially increase this effect. In no way can it be attained with testosterone alone, even in high doses.

The former findings of Tschopp [1936] that there was no difference between the effect of testosterone and that of its acetate is explained by the fact that at that time an older oil, probably rich in activators, was used. As a result of this, a wrong impression was obtained of the activity of testosterone, whilst on the other hand the effect of the acetate was not influenced. It would be desirable to employ inert solvents such as paraffin oil for elaborating these tests; however, we generally refrained from using such inert oils since in clinical practice only fatty oils are used.

Second method using two injections with an interval of 5 days. The above experiments show the effect of the esters only on the 11th day following the beginning of the test, but do not give any idea as to the time course of the action. In order to ascertain this, relatively high doses of 1 mg. each, dissolved in 1 ml. sesame oil, were injected into castrated rats on the 1st and 6th days of the experiment. Some of the animals were killed and examined on the 11th day, others at further constant intervals of 5 days.

The effects on the seminal vesicles and prostates are shown in Figs. 4 and 5. The course of the effect on the other organs weighed (preputial glands, Cowper's glands) is quite similar. The general picture of the results obtained with the ten-injections method is changed to a marked degree by the new method. Now on the 11th day the effect of testosterone has already disappeared whilst that of the esters is more or less prolonged. There is a striking difference between the formate and acetate on the one hand, whose effects are already very small on the 11th day, and on the other hand the neighbouring higher esters, beginning with the propionate, which show very high activities. Propionate attains its optimum effect at the 11th day, or earlier, butyrate at the 16th and *n*-valerate only at the 21st day. In the last case especially the increase of the effect is retarded; on the other hand it is the curve of this ester which attains the highest level. The *iso*compounds, such as *iso*butyrate and especially *iso*valerate, have distinctly weaker effects than the corresponding normal esters. The activities of *n*-decanoate and benzoate are considerably retarded and diminished but are therefore of relatively long duration. In this test stearate and palmitate showed no effect.

Third method using a single injection. In order to obtain a still clearer picture of the development of the effect with time we administered in another series of experiments on the 1st day a single dose of 2 mg., dissolved in 2 ml. of sesame oil. We began with examination of the rats already about the 4th day, using at least three animals for the determination of each value. The results regarding the seminal vesicles and prostates are given in Figs. 6 and 7. The effect of testosterone compared with that of the esters is again remarkably slight. As in the foregoing test, the formate and acetate are as effective as testosterone plus 200 mg. of palmitic acid. Their maximum effects are observed already on the 6th day. With propionate, butyrate and valerate, the maximum attains a much higher level than with the lower esters. The whole activity lasts at least 2–4 weeks. In spite of this more intensive and protracted action of these highly active esters, the rate of increase of the effect compared with formate and acetate is not diminished. This is, however, the case with the less effective esters of testosterone, like *n*-decanoate and benzoate.

We found that the testosterone esters act not only on the sex organs of the castrated rats but also on those of the uncastrated rats; we shall return to this question at a later date.

Besides the testosterone esters we examined under the new test conditions on the rat (and also on the capon's comb) a considerable number of esters of other compounds with male hormone activity, for instance the esters of dihydrotestosterone (androstane-3-one-17-*trans*-ol) and androstanediol. These results will also be published in due course.

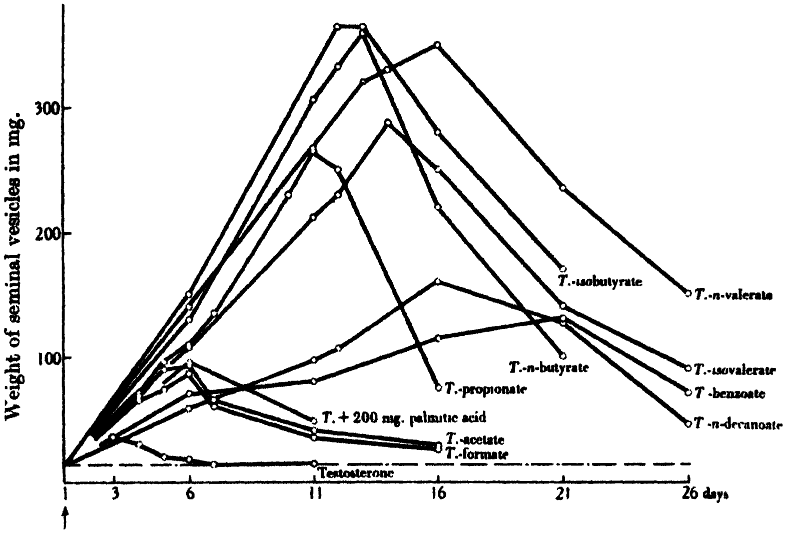

Fig. 6. Effect of testosterone esters on the weight of seminal vesicles (method using a single injection).
T = Testosterone.

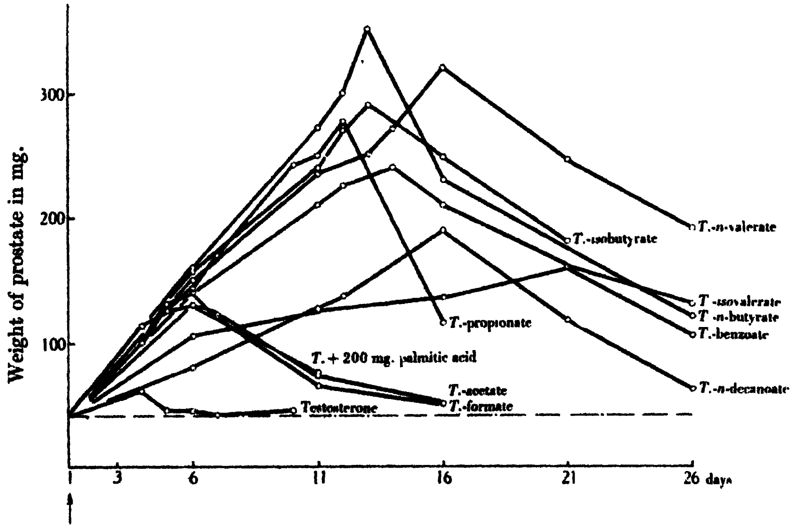

Fig. 7. Effect of testosterone esters on the weight of prostate (method using a single injection).
T = Testosterone.

II. *The ratio between the activities of testosterone esters on the seminal vesicles and on the prostate and between those on capons and rats.*

In Fig. 8 the ratio between the activities of testosterone esters, of free testosterone and of testosterone + palmitic acid on the seminal vesicles and

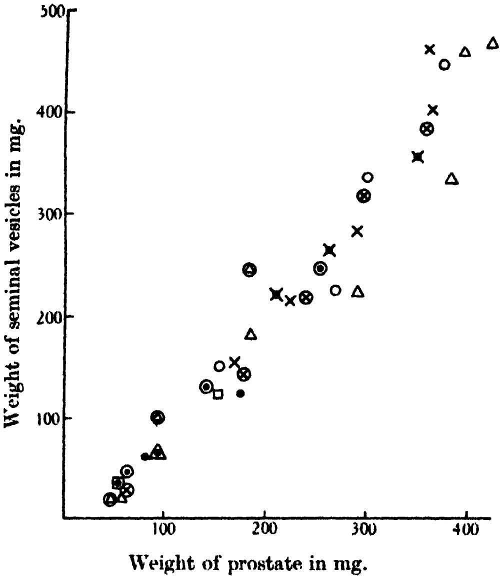

Fig. 8. The ratio between the activity of testosterone esters on the seminal vesicles and on the prostate.

× *T.*-formate.
⊗ *T.*-acetate.
○ *T.*-propionate.
△ *T.*-*n*-butyrate.
◬ *T.*-*iso*butyrate.
□ *T.*-*n*-valerate.
▣ *T.*-*iso*valerate.
◉ *T.*-*n*-decanoate.
◎ *T.*-benzoate.
• Testosterone (= *T.*).
ж *T.* + daily 50 mg. palmitic acid.

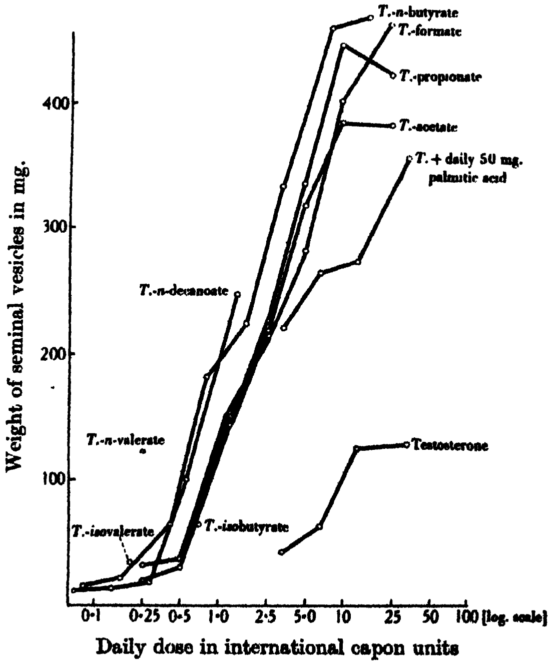

Fig. 9. The ratio between the activities of testosterone esters on the seminal vesicles of the rat and on the capon comb.
T = Testosterone.

Table III.

	Daily dose (γ) dissolved in 0·5 ml. sesame oil	Without palmitic acid Weight of the organs in mg.				Number of rats	With 50 mg. palmitic acid daily Weight of the organs in mg.				Number of rats
		Seminal vesicles	Prostate	Cowper's glands	Preputial glands		Seminal vesicles	Prostate	Cowper's glands	Preputial glands	
cis-Androsterone acetate	500	14	32	—	37	2	12	40	50	46	2
cis-Androsterone benzoate	500	13	39	40	45	2	12	38	42	45	2
trans-Dehydroandrosterone acetate	3000	56	92	117	178	2	—	—	—	—	—
	2000	36	72	75	86	2	43	70	81	90	2
	500	13	43	42	78	2	13	40	46	96	2
Testosterone acetate	200	383	357	228	136	3	302	334	220	149	3
	100	286	234	227	98	3	250	322	210	111	3
	50	240	241	195	86	4	210	279	215	96	4
	25	142	180	175	75	4	134	179	131	64	4
	10	30	62	83	49	4	33	70	119	46	4
Testosterone propionate	50	225	270	190	89	4	207	253	251	119	2
Testosterone *n*-butyrate	50	181	185	190	73	4	215	214	180	86	2
Testosterone *n*-decanoate	50	15	38	50	45	4	19	43	54	60	2
Testosterone benzoate	200	140	142	136	80	2	210*	150*	148*	80*	2*
Androstane-3-*cis*-17-*trans*-diol 3-benzoate	1000	77	90	98	60	2	—	—	—	—	—
	500	26	51	56	47	2	45	85	80	80	2
	200	—	—	—	—	—	30	61	72	55	2
Androstane-3-*cis*-17-*trans*-diol 17-benzoate	500	49	83	71	72	2	—	—	—	—	—
	200	—	—	—	—	—	25	47	63	76	2

* Values obtained with brassidic acid.

prostate, according to the 10-days test, is shown graphically. With the exception of the benzoate all the active esters in higher doses produce seminal vesicles which are heavier than the prostate, as is the case in the normal adult rat. With testosterone + palmitic acid equality of weights is reached; with testosterone alone in all doses the seminal vesicles are lighter than the prostate, as is the case in the infantile or castrated rat. When comparing the maximum weights of seminal vesicles and prostates of rats obtained according to the second and third test methods (repeated and single injection), it is remarkable that only by using highly active esters, such as propionate etc., can a weight of the seminal vesicles surpassing that of the prostate be obtained.

It was recently shown by Deanesly & Parkes [1936, 2] that testosterone is about as active on the seminal vesicles and prostates as androsterone when given in doses containing the same number of capon units; thus there is still a considerable difference between the qualitative actions of testosterone and testicular extracts. As will be seen from Figs. 9 and 10 (rat test using ten injections) the esters of testosterone and also testosterone + palmitic acid appear to act much more favourably in this respect, so that similar conditions are attained as by using testicular extracts.

III. *The influence of acids on the activity of esters.*

Table III gives particulars of the results of our experiments to determine the influence of palmitic or brassidic acid on the effects of the esters of testosterone, androsterone, *trans*-dehydroandrosterone and androstanediol in the 10-days test. Pronounced increases of activity were only observed with testosterone benzoate and androstanediol benzoate (but not with androsterone benzoate).

Table IV contains results of experiments made to determine the influence of the solvent on the effects of testosterone and testosterone acetate, with and without an activator, on the seminal vesicles and prostate. Testosterone in 50 %

Table IV.

	Daily dose γ	Weight of glands in mg. Seminal vesicles	Weight of glands in mg. Prostate	Number of rats
Testosterone in sesame oil	50	42	66	4
Testosterone in glycerol 50 %	500	20	—	2
	50	16	46	2
Testosterone in glycerol + daily 50 mg. ricinoleic acid	50	221	205	2
Testosterone in paraffin oil	50	15	44	2
Testosterone in paraffin oil + daily 50 mg. palmitic acid	50	126	145	2
*Testosterone acetate in sesame oil	200	383	357	2
	50	218	241	8
*Testosterone acetate in sesame oil + daily 50 mg. palmitic acid	200	302	334	2
	50	210	279	2
Testosterone acetate in paraffin oil	200	252	293	2
	50	65	105	2
Testosterone in glycerol 50 %	125	45	72	2
	50	12	36	2
Testosterone acetate in glycerol + 50 mg. ricinoleic acid	125	215	283	2

* See also Table III.

glycerol, as well as in paraffin oil, is almost ineffective; the addition of acids and particularly of 50 mg. ricinoleic acid to the glycerol solution activates it to a striking degree. On the other hand we had formerly found that an oily solution of testosterone is only slightly influenced by the addition of ricinoleic acid. In the experiment with glycerol an unsaturated hydroxy-acid was chosen instead of palmitic acid since the latter is only slightly soluble in glycerol.

Testosterone acetate dissolved in paraffin oil is less effective than in sesame oil. It dissolves badly in aqueous glycerol and its effect is only very slight therein. In this instance also the effect can be increased almost to the normal level by the addition of 50 mg. ricinoleic acid. Thus the remarkable fact emerges that the effect of testosterone acetate, when dissolved in aqueous glycerol, is increased by the addition of an activator, but that such an addition does not increase the effect when testosterone acetate is dissolved in sesame oil. Further investigations will be required in order to elucidate this result.

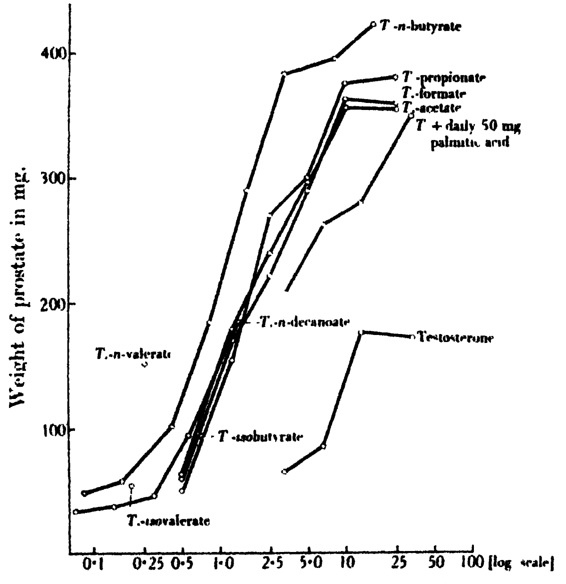

Fig. 10. The ratio between the activities of testosterone esters on the prostate of the rat and on the capon comb.

T = Testosterone.

IV. *The activation of testosterone by other hormones.*

For the sake of completeness it should be mentioned that the effect of testosterone can also be enhanced by addition of other hormones. The reciprocal influence of the anterior lobe of the pituitary gland is well known. After the Amsterdam investigators had described the "paradoxical" influence of the female hormone on the male sexual organs, Korenchevsky [1935; Korenchevsky & Dennison, 1935; Korenchevsky *et al.* 1935, 2], Tschopp [1936] and Steinach *et al.* [1936; see also Clauberg, 1936; Petterson, 1936] recently demonstrated in rats the reinforcing influence of oestrone and its derivatives on the effect of the male hormones.

V. *The question of the standardization of the male sex hormones.*

At the "Second conference on the standardization of sex hormones"[1] appointed by the League of Nations Health Organization, and held during the summer of 1935, a standard was fixed for male sex hormone preparations. According to this the unit of activity was defined as the activity of 0·1 mg. of the standard androsterone preparation as measured by a specific biological reaction. In the opinion of the members of the conference, the capon's comb test was at that time the only specific test which allowed comparison with the standard. Attempts were recommended to improve the standardization by using mammals.

The reference to the specificity of the capon's comb test is, according to our investigations, warranted, since it is less suceptible to influences of the medium than the rat test; in particular no activating effect has so far been observed on the comb. Even comparison of the standard with esters having protracted action is possible, provided that only the optimum effect is considered and that the demand for simultaneous reading of the standard effect and that of the preparation is abandoned as in our experiments. In this manner it is possible to avoid the trouble of having to choose a new standard for every ester.

It ought to be possible to standardize not only the intensity but also the duration of the effect, for example, by determining the ratio of the number of days in which the maximum effect is attained, on the one hand by the stipulated quantity of the standard and on the other by the compound to be tested. The notion that two preparations, showing the same activity when expressed in international units, must agree in all their pharmacological properties is not justified, since it is known that the relation between the effects on the capon's comb and on the rat can also be quite different when various preparations are tested. Even the relationship between the effects on the seminal vesicles and on the prostate can vary to a considerable extent [see also Deanesly & Parkes, 1936, 2]. Therefore, evaluation in units always relates only to a narrowly limited property and no indication is given as regards other actions; if it is desired to standardize these, other tests and new units will be needed.

The question, however, whether the capon test is sufficient for the characterization of male sex hormone compounds, must be answered in the negative; testosterone benzoate for example is practically ineffective in this test, but effective in the rat.

In view of the above, the following conclusions may be drawn in connexion with the testing of male sex hormone preparations:

(1) In addition to capon units, rat units should be introduced [see e.g. Korenchevsky *et al.* 1935, 1; 1936].

(2) The comparison can only refer to a narrowly limited property.

(3) For this purpose, as the basis for comparing the intensities of effect, only the maximum effects produced by the standard and the preparation should be selected, without regard to the time when the maxima appear. The duration of the effect affords a special characterization.

(4) The methods used for the tests must be exactly standardized.

The characterization of the effect of a preparation in units is only a temporary expedient. Every substance is really only comparable with itself. Nevertheless the definition of generally accepted standards is of great practical importance because it facilitates a quick evaluation of the most varied preparations in an accepted measure.

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[1] This conference was attended by one of us (K.M.).

SUMMARY.

1. Eleven testosterone esters (nine of them new), especially with acids of the aliphatic series, have been prepared and examined as regards their effects.

(*a*) The lowest esters (formate, acetate and propionate) are the most effective on the capon's comb. The longer the carbon chain of the fatty acid residue, the more protracted is the effect, but meanwhile the intensity decreases rapidly. Palmitate and stearate, as well as benzoate, are practically ineffective.

(*b*) In the rat test (seminal vesicles, prostate etc.) the lower esters of testosterone are generally many times more effective than testosterone alone. For the first time in addition to the intensity the duration of activity of male hormone preparations was also tested on rats; in this way it was shown that in both respects testosterone formate and acetate give results approximately similar to those obtained with testosterone plus an activator. However, higher esters, from propionate upwards, proved to be much more effective. According to the test the activity reaches maximum intensity and duration in the cases of the *n*- and *iso*-butyrates or *n*-valerate. When we pass to still higher esters, especially to *n*-decanoate and benzoate, the rise in effect is already greatly retarded and its maximum diminished. Testosterone palmitate and stearate are ineffective.

2. When the capon and rat tests are considered simultaneously testosterone propionate shows an especially favourable total action. Comparing testosterone, testosterone + palmitic acid (or testosterone acetate) and testosterone propionate by the method using one injection of 2 mg., the maximum increases in the weight of the seminal vesicles of castrated rats obtainable are in the proportion of approximately 1:4:12.

3. According to the different test methods on the rat, only by using esters of testosterone, like the propionate etc. is it possible to produce in the castrated infantile rat seminal vesicles heavier than the prostate, as is the case in the normal adult rat. Using free testosterone the prostate remains always heavier than the seminal vesicles, as is the case in castrated and infantile rats.

4. Testosterone esters are relatively more effective on rats than on capons, as is also the case with natural testicular extracts, and differ in this respect from androsterone and even from testosterone. In the case of the lower esters, up to the propionate, this favourable relation is due to a high activity on rats, not to a low effect on capons.

5. The effects of the esters of the testosterone and androsterone series are in general not increased by addition of acids when dissolved in fatty oils. The specially low activity of testosterone acetate in 50 % glycerol however is nearly brought to the normal by the addition of ricinoleic acid.

6. The recommendation to introduce besides capon units also rat units is again put forward. The methods of standardization should be accurately defined. Instead of simultaneous comparison between the standard and the preparation to be tested, comparison of the maximum intensities of the effects independently of their time of occurrence is recommended. Intensity and duration of the effect cannot be fixed by one unit alone. The duration of the effect should be characterized separately.

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Note added 9 October 1936. Our results regarding the activities of the testosterone acetate and propionate have been fully confirmed in the meantime by Parkes [1936] *Lancet*, ii, 674. This author has also made other valuable observations with regard to these esters.

CCLXXVIII. CEREALS AND RICKETS.[1]

VIII. THE HYDROLYSIS OF PHYTIN IN THE INTESTINE.

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INTEREST in the availability of phytin-P was stimulated by the report of Bruce & Callow [1934] that the rachitogenic manifestation of cereals was effected by the low availability of cereal P. McCance & Widdowson [1935] found that in man as much as one-half of the phytin-P was unavailable. The possible importance of this was revealed by their analyses which showed that phytin-P accounted for 46·4–66·0 % of the total P present in the common cereal grains such as wheat, maize and rolled oats.

Recently it was shown by Templin & Steenbock that immature maize [1933, 1] and germinated autolysed maize [1933, 2] were far less rachitogenic than the matured kernel. This difference was demonstrated by Lowe & Steenbock [1936] to be paralleled by an increase in inorganic P. Phytin, isolated from wheat bran, when fed as such was a poor source of phosphorus in a rachitogenic ration. The basal ration used in these experiments was Ration 2965 composed of 76 % yellow maize, 20 % wheat gluten, 3 % $CaCO_3$ and 1 % NaCl.

In view of these results, it seemed desirable to determine if phytin-P were generally unavailable or if its unavailability were but an artefact produced by an unusual diet. The high content of calcium in Ration 2965 might conceivably have altered relations so as to present an unusual picture of phosphorus availability. For this determination additional experiments have now been carried out using adult rats as the experimental animals.

EXPERIMENTAL.

Metabolism of phytin and inorganic P.

Both high and low calcium rations were fed. Four female rats weighing approximately 200 g. were used for each ration. Individual collections and analyses of excreta were made for two 4-day periods for each animal. All animals were kept on their respective rations for a preliminary period of not less than 4 days, but always until their food consumption was normal and their weights showed no decline. Food consumption was equalized so far as possible.

The urines were collected under toluene. The faeces were preserved by allowing them to drop into a collection chamber in which the air was saturated with vapour of formaldehyde. They were dried overnight at 100°, then finely ground for analysis. Both faeces and urine were analysed for inorganic and total P using essentially the method of Fiske & Subbarow [1925].

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[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

The low calcium basal ration consisted of yellow maize 76, wheat gluten 20, sodium chloride 1, or, in other words, Ration 2965 from which the $CaCO_3$ had been omitted. Analysis showed it to contain 0·058 % Ca and 0·28 % P. When phosphorus was given it was added as disodium hydrogen phosphate or as phytin. Calcium was added as the carbonate. The phytin was prepared from wheat bran. It was free from inorganic P. It was mixed, as a fine powder, directly into the basal ration. The disodium hydrogen phosphate was added in solution, by dissolving it in a minimum of water and then evaporating it on the ration. The P supplements provided five times the amount of phosphorus present in the basal ration. The P supplements were made ample to reduce the proportionate effect of the non-phytin P compounds of the basal ration.

The most significant result from our feeding experiments when phytin was the source of P was the marked diminution in its hydrolysis effected by the presence of $CaCO_3$. The urinary P dropped to mere traces and the total excretion of inorganic P was reduced to about one-fifth of its former value. The P, in this case, was excreted entirely through the faeces and almost all in organic combination. In the absence of $CaCO_3$, phytin was hydrolysed to a small but very definite extent, as shown by the rise in urinary P over that resulting from the feeding of the basal ration alone.

Table I. *Effect of $CaCO_3$ on the retention and excretion of phytin-P.*

Averages of 8 analyses on 4 rats over two 4-day periods.

		Total P			Urinary P		Faecal P		
No.	Ration	Intake mg.	Output mg.	Balance mg	Total mg.	% of output	Inorganic mg.	Total mg.	% of output
1	Basal	96	83	+ 13	45	54	21	38	46
2	Basal + phytin	493	450	+ 43	112	25	173	338	75
3	Basal + phytin + $CaCO_3$	486	450	+ 36	Trace	Trace	56	450	100
4	Basal + Na_2HPO_4, $12H_2O$	544	411	+133	347	84	37	64	16
5	Basal + Na_2HPO_4, $12H_2O$ + $CaCO_3$	510	424	+ 86	185	44	218	239	56

When disodium hydrogen phosphate was fed in place of phytin, the P was excreted, as was to be expected, chiefly through the urine. The addition of $CaCO_3$ also caused a marked reduction in the urinary P although this condition was not entirely analogous to that obtained when $CaCO_3$ was fed with phytin. In the case of the phytin-$CaCO_3$ ration, the urinary P was reduced to traces because of the greatly diminished hydrolysis of phytin as shown by the lowered inorganic P content of the faeces as well as of the urine. However, when $CaCO_3$ was given with disodium hydrogen phosphate, the total excretion of inorganic P remained practically the same, $CaCO_3$ presumably acting merely by reducing the absorption of P from the intestinal tract.

A consideration of the P balances on these rations also reveals some interesting information. It is apparent that the greatest retention of P occurred on the phosphate ration, yet even in the presence of $CaCO_3$ the retention of P was higher than it was on the phytin ration alone. Small differences in P retention probably would not be revealed because of the use of adult animals, but these differences appear to be significant.

From these data, it would seem justifiable to deduce two important generalizations; first, that phytin-P is not completely unavailable to the rat, and secondly that the small amount which might be available is rendered almost completely unavailable by the presence of $CaCO_3$.

Effect of other substances on the hydrolysis of phytin.

In view of the pronounced inhibitory effect of $CaCO_3$ on the hydrolysis of phytin, it appeared desirable to investigate the effect of other salts which have been shown to increase the severity of experimental rickets to varying degrees. These studies were limited to following the distribution of total and inorganic P in the faeces.

Table II. *Effect of various compounds on the hydrolysis of phytin*

		Faeces		
No.	Ration	Inorganic P %	Total P %	Inorganic % of total
6	Basal + phytin	2·9	6·7	43·3
7	Basal + phytin + 3 % $CaCO_3$	0·86	7·8	11·0
8	Basal + phytin + 3 % $MgCO_3$	0·89	7·3	12·2
9	Basal + phytin + 3 % $SrCO_3$	1·2	7·9	15·2
10	Basal + phytin + 3 % $BeCO_3$	1·2	7·8	15·4
11	Basal + phytin + 3 % Al_2O_3	2·3	6·3	36·5
12	Basal + phytin + 3 % Fe_2O_3	3·0	6·0	50·0

The rations were prepared by adding 3 % $MgCO_3$, $SrCO_3$, $BeCO_3$, Al_2O_3 or Fe_2O_3 in place of the $CaCO_3$. The consumption of the rations was kept relatively constant. The data presented in Table II show that $MgCO_3$, $SrCO_3$, $BeCO_3$ all produced an effect similar to $CaCO_3$ in inhibiting the hydrolysis of phytin as judged by the percentage of the total P of the faeces excreted in the inorganic form. The total amounts of P excreted remained practically constant except in the case of $BeCO_3$ where a lower food intake led to a corresponding drop in the P excretion. Al and Fe oxides caused only slight differences. Data were not obtained on the urinary P in these cases, so a comparison of the P balances could not be made. However, in view of the uniformity in P distribution in the faeces on all the rations, it appears likely that the modes of action of the other salts resembled that of $CaCO_3$.

Our attention is necessarily again directed to the means whereby the hydrolysis of phytin is effected. The intestinal mucosa is well known to be rich in a phosphatase which hydrolyses glycerophosphate and a wide variety of phosphoric acid esters. Armstrong [1935] has recently found that dog faeces are an excellent source for the preparation of a phosphatase; but Plimmer [1913] reported that extracts from the intestinal mucosa do not contain a phytin phosphatase—a fact which we have substantiated for both the chick and the rat. On the other hand, we have found both a glycerophosphatase and a phytin phosphatase present in faeces and in the contents of the small and large intestines of the rat. This faecal phytase showed an optimum activity distinctly on the acid side of neutrality.

Acid phosphatases have been reported by a number of investigators. Adler [1915] discovered a phytase in malt which showed an optimum activity at *p*H 5·4, and Kay & Lee [1931] reported a phosphatase in soy beans having an optimum *p*H at 5·2. Roche [1931] showed that the red blood corpuscles contained an acid phosphatase with an optimum *p*H between 6·0 and 6·8 and Davies [1934] found that extracts of both spleen and liver contained an acid as well as an alkaline phosphatase. The acid phosphatases, whether acting on phytin or glycerophosphate as a substrate, are quite distinct from the dominant phosphatase of mammalian tissues [Kay, 1932]. In considering the mode of hydrolysis of phytin in the intestine, it is obvious that the phytase of the flora of the gastro-intestinal tract as well as the phytase ingested with the food warrant intensive investigation.

The availability of phytin-P in different cereals.

In line with the premise that the gastro-intestinal environment can affect the hydrolysis of phytin, the question arose as to whether cereals might not differ in the extent to which they promote this action. The activity of the intestinal flora produced on one cereal might be substantially different from that produced on another.

To study this question, the yellow maize component in our basal diet was replaced in one case by rolled oats and in another by whole wheat. Each was fed with and without $CaCO_3$ to adult female rats. The rats were kept on their respective rations for a preliminary period of 5 days. The urines were not collected because we were concerned primarily with the comparative hydrolysis of the phytin in the rations and not with differences in P retentions. With our adult rats, retention of phosphorus would naturally be low.

Table III. *Effect of varying the cereal component in Ration 2965.*

		Faeces		
No.	Modification of Ration 2965	Inorganic P %	Total P %	Inorganic % of total
13	No modification	0·71	2·13	33·5
14	Same as 13 minus $CaCO_3$	0·60	1·25	47·8
15	Rolled oats substitued for yellow maize	0·83	2·44	33·8
16	Same as 15 minus $CaCO_3$	0·77	1·68	45·9
17	Whole wheat substituted for yellow maize	0·69	2·06	33·7
18	Same as 17 minus $CaCO_3$	0·45	1·11	40·1

The results are presented in Table III. They afford no basis for assuming any differential action of maize, rolled oats or whole wheat. They do offer additional evidence in support of the fact that $CaCO_3$ has a definite inhibitory action on the hydrolysis of phytin. This action was noted without exception with each of the cereals studied.

Various other modifications of the basal ration which might be expected to change the intestinal flora and so alter the degree of hydrolysis of phytin were made. Lactose, lard and vitamin D were added with and without $CaCO_3$. The results from the effect of these additions are presented in Table IV. They show

Table IV. *Effect of various modifications of Ration 2965.*

		Faeces		
No.	Modification of Ration 2965	Inorganic P %	Total P %	Inorganic % of total
19	No modifications	0·71	2·13	33·5
20	Same as 19 minus $CaCO_3$	0·60	1·25	47·8
21	Plus 1250 U.S.P. units vitamin D/day	0·57	1·75	32·6
22	Same as 21 minus $CaCO_3$	0·45	0·74	60·8
23	Plus 10% lactose	0·77	1·95	39·5
24	Same as 23 minus $CaCO_3$	0·35	0·72	48·6
25	Plus 30% lard	0·51	1·54	33·1
26	Same as 25 minus $CaCO_3$	0·26	0·59	44·0

that the percentage of the total P excreted in the faeces as inorganic P was uniform with all rations containing $CaCO_3$. In every case, these values were lower than those from corresponding rations without $CaCO_3$. The vitamin D supplement in the absence of $CaCO_3$ gave an unusually high proportion of total P present as inorganic P. This value stands as an isolated observation.

The possible relation of our findings on the hydrolysis of phytin to rachitogenic diets used for assay purposes bears mention. Variability in the utilization of phytin-P may be a factor of no small significance in explaining the irregularities reported in the literature in the production of experimental rickets [Harris & Bunker, 1931; 1934; 1935]. It is obvious that our rachitogenic diets as used for assays should contain their P in the form of compounds of invariable nutritive value. Whether this can be achieved in the presence of a substantial content of organic P compounds appears doubtful.

SUMMARY.

1. Phytin when fed to rats on a low Ca and low P ration was hydrolysed to a substantial though incomplete degree.

2. The hydrolysis of phytin was greatly diminished by including 3 % $CaCO_3$ in the ration. This same effect was observed with the use of various other salts known to have a rachitogenic action.

3. The substitution of whole wheat or rolled oats for yellow maize in the basal ration or the addition of lard or lactose effected no significant change in the excretion of P.

4. The role of intestinal flora must be given more consideration in the solution of problems in nutrition than has hitherto been the case.

The authors wish to express their appreciation to Harold A. Orlove for assistance in the analytical work.

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CCLXXIX. THE CARBOHYDRATE METABOLISM OF THE KIDNEY.

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The chemical changes leading to the formation of lactic acid from carbohydrate in animal tissues have been extensively investigated. The evidence now seems to be fairly complete for the sequence of chemical reactions, embodied in the well-known scheme of Embden *et al.* [1933], leading to lactic acid formation in normal skeletal muscle; nevertheless it is believed by some that the older methylglyoxal theory is not yet entirely discredited and that it must be regarded as one which represents an alternative path of glycolysis in muscle. Lactic acid formation in frog heart has been studied extensively by Gaddie & Stewart [1934] and by others; Ashford & Holmes [1929] and Ashford [1933; 1934] have made similar studies using brain tissue. The conclusion reached was that brain tissue may form lactic acid in either of two ways, namely by the breakdown of glycogen with the intervention of phosphate and by the degradation of glucose without the participation of phosphate. The work of Jost [1934] on the formation of lactic acid in ox kidney brei led him to conclude that carbohydrates in the kidney are transformed into lactic acid by the same series of reactions as that postulated by Embden for muscle. Jost demonstrated the anaerobic formation of lactic acid in kidney brei from glyceraldehydephosphoric acid, from glyceraldehyde, from a mixture of glycerophosphoric acid and pyruvic acid and from a mixture of glycerophosphoric acid and phosphoglyceric acid. Under the conditions of his experiments pyruvic acid, when added alone to kidney brei, was reduced to lactic acid but the amount of the latter was much less than that formed from a mixture of pyruvic acid and glycerophosphoric acid. He was unable to decide whether the kidney possesses an alternative mechanism by which lactic acid may be formed from carbohydrate without the formation of intermediary phosphorylated compounds.

The present investigation was designed to throw some further light on the mechanism or mechanisms controlling lactic acid formation from carbohydrate by surviving kidney cortex. The paper deals with (*a*) the effects of fluoride, cyanide and iodoacetic acid on both lactic and phosphoric acid production in the excised kidney; (*b*) the glycogen content of the kidney; (*c*) the effect of arsenate on carbohydrate breakdown to lactic acid by kidney tissue; (*d*) pyruvic acid as an intermediary in kidney glycolysis.

Methods.

Normal male rabbits, averaging 2·35 kg., were used. Unless otherwise stated, the animals were killed by a sharp blow on the back of the neck, the kidneys were excised as quickly as possible, and the cortex was rapidly clipped off. Approximately equal portions (2–3 g.) of the tissue were dropped into the following weighed vessels. The time elapsing between the death of the animal and the "initial value" averaged 1–1·5 min. The experimental vessels were set up as follows:

I. "Initial value." 50 ml. weighing bottle containing 5 ml. cold 25% trichloroacetic acid. The lactic acid found in this mixture was termed the "initial value".

II. 50 ml. weighing bottle containing 5 ml. suspension medium.

III. 50 ml. weighing bottle containing 5 ml. isotonic saline.

The vessels were re-weighed and the weights of tissue calculated. Vessels II and III were then incubated at 37·5° for 2 hours without antiseptic.

Immediately after samples Nos. II and III had been placed in the water-bath, the contents of No. I were finely ground with sand. The thin brei was transferred quantitatively to a 25 ml. cylinder, made up to known volume (15 or 20 ml.) with 6 % trichloroacetic acid and filtered through a Whatman No. 30 filter-paper into a small flask which was stoppered tightly and placed in the refrigerator.

At the end of the incubation period samples II and III were removed from the water-bath and 2 ml. of 25 % trichloroacetic acid added to each. The contents of each tube were then treated as described for sample I. The three filtrates thus obtained were analysed for lactic and phosphoric acids.

The lactic acid was extracted with ether as follows. To 5 ml. of the protein-free filtrate in a 50 ml. glass-stoppered cylinder were added 2·5 g. of ammonium sulphate, 2 ml. of 85 % phosphoric acid and five volumes of purified ether; the contents of the cylinder were shaken vigorously for 5 min. The ethereal layer was drawn off with a 15 ml. rubber-bulb pipette and filtered through a Whatman No. 50 paper into a 500 ml. flask. The above process was repeated five times; the stopper, pipette, filter-paper and funnel were washed with small portions of ether which were added to the main extract. Two ml. of 10 % sodium hydroxide solution were then added and the ether was distilled. After removal of the last traces of ether from the flask by aeration, the residue was dissolved in water and the solution made up to volume in a 50 ml. flask.

Lactic acid estimations were then carried out in duplicate on 15 ml. of this solution according to the method of Friedemann *et al.* [1927]. The concentration of sulphuric acid was reduced and that of manganese sulphate increased in accordance with the suggestion of Friedemann & Kendall [1929].

The colorimetric method of Briggs [1922] was used to determine inorganic phosphorus in the trichloroacetic acid filtrates. Total acid-soluble phosphorus in these filtrates was estimated by the method of Kay & Byrom [1927] and the precautions emphasized by Kay [1931] were carefully observed.

EXPERIMENTAL.

It was decided in the beginning to investigate the effect of sodium fluoride on lactic acid production and on phosphate liberation in the excised kidney, since at that time (1932) it was agreed by most workers in the carbohydrate field that, for skeletal muscle at least, the inhibition of lactic acid formation by sodium fluoride was due to the stabilization of hexosephosphate.

It was found that lactic acid production was completely inhibited by $M/5$ fluoride but there was no marked inhibition of phosphate liberation. Moreover Kay (private communication) has shown that the rate of breakdown of the naturally occurring phosphoric ester in the kidney is entirely independent of that of the production of lactic acid. These observations support the view that the precursor of lactic acid in the kidney is a non-phosphorylated compound. In this connexion it is of interest that Ashford & Holmes [1929] and Ashford [1933; 1934] have shown that while phosphates may play a part in the formation of lactic acid from glycogen, the major portion of the lactic acid production of surviving brain tissue is independent of the intervention of phosphate.

Experiments were next performed to determine the effect of cyanide on the two processes and it was found that $M/100$ cyanide at pH 7·0 has no appreciable effect on phosphate liberation and lactic acid production.

The effect of sodium iodoacetate at pH 7·0 on the liberation of inorganic phosphate and on lactic acid formation in the kidney was next studied. Lohmann [1932], Jowett & Quastel [1933] and Dickens [1933] have shown that in skeletal muscle iodoacetate inhibits glyoxalase activity by interacting with glutathione which functions as the coenzyme of glyoxylase. Since this enzyme is known to be present in both skeletal muscle and kidney and since both of these tissues possess the ability, under certain experimental conditions, to convert hexosephosphate into methylglyoxal, it was reasonable to suspect that lactic acid formation normally proceeds by way of methylglyoxal and that iodoacetate blocks glycolysis at the methylglyoxal stage. However the well-known experiments of Lohmann who demonstrated the formation of lactic acid from glycogen by muscle extracts devoid of glutathione revealed the possibility of another series of chemical changes, representing at least an alternative route, leading to the formation of lactic acid in muscle. The changes embodied in the scheme of Embden *et al.* [1933] represent such a route. It has been shown [Meyerhof, 1935] that iodoacetate inhibits the oxidation of triosephosphate to phosphoglyceric acid by yeast preparations; furthermore Embden & Deuticke [1934] observed that muscle poisoned by bromoacetate is no longer able to convert hexosediphosphate into a difficultly hydrolysable ester or to transform pyruvic acid into lactic acid even in the presence of glycerophosphate as hydrogen donor. They concluded that the halogenacetic acids may inhibit glycolysis by preventing these oxidation-reduction transformations.

The results of several experiments are set out in Table I. It will be seen that iodoacetate (pH 7·0) stops all lactic acid formation in the excised kidney. This drug has, however, no significant inhibitory effect upon the liberation of phosphate. These observations are in conformity with the view that the main path of carbohydrate degradation to lactic acid by kidney tissue is one which does not involve the participation of inorganic phosphate. It is of interest to note that in every case except Exp. A of Table I the lactic acid content of the tissue decreased during incubation in the presence of iodoacetate. These results are in accord with those of Haldi [1932] who noted a disappearance of lactic acid in kidney tissue incubated in the presence of iodoacetate, but not in brain tissue under comparable conditions. A loss of lactic acid in excised muscle was

Table I. *Effect of iodoacetate on phosphate liberation and lactic acid production by kidney cortex incubated at 37·5°.*

All values are expressed as mg. per 100 g. of freshly chopped tissue.

	Time of incubation hours	Free P				Total acid				Lactic acid			
		A	B	C	D	A	B	C	D	A	B	C	D
Tissue plus 25% trichloroacetic acid "Initial value"	0	28	43	29	33	112	119	96	114	78	101	72	89
Tissue plus sodium iodoacetate* (pH 7·0)	2	81	104	64	91	123	130	101	133	82	67	51	52
Tissue plus isotonic saline	2	80	104	71	86	125	132	124	134	127	—	125	152

* Concentration of iodoacetate in Exps. A and B, 0·01 M; and in C and D, 0·02 M.

observed by Lundsgaard [1930] and by Meyerhof & Himwich [1924] who attributed the loss to oxidation. The observed loss of lactic acid in kidney cortex might be likewise explained.

In search for the source of lactic acid, the first which suggested itself was glycogen. A review of the literature relating to the glycogen content of this organ has been made by Irving [1928]. Irving determined the glycogen content of rabbit kidney cortex but views his own results with suspicion, inasmuch as he believes, that they are affected to a large extent by non-sugar substances. It was necessary, therefore, to reinvestigate the problem.

Estimation of glycogen.

The estimations of the glycogen content of the kidney cortex were made on material obtained from normal male rabbits anaesthetized with amytal. 45 mg. of amytal per kg. of body weight were dissolved in 3 ml. of water and injected intravenously. When anaesthesia was complete, one kidney was exposed without undue handling and carefully removed. Approximately 3 g. of cortex were rapidly clipped off into a weighed 15 ml. pyrex centrifuge-tube equipped with a rubber stopper and a straight glass condenser and containing 2 ml. of hot 60 % potassium hydroxide. The time taken for this operation averaged 18 sec. After the tissue had been thoroughly mixed with the alkali the tube was returned to the boiling water-bath. The second kidney was treated similarly.

After 30 min. in the water-bath the tubes were cooled, re-weighed and returned to the bath for a total of 3 hours, after which they were removed and allowed to cool slightly but not to form a gel. Absolute alcohol was added until the final concentration was 66 %. The vessels were stoppered, shaken gently and allowed to stand overnight in the refrigerator. The material was centrifuged at 2500 r.p.m. for 15 min. and the supernatant fluid was carefully removed from the precipitate with a fine capillary pipette attached to a pump. The precipitate was washed by triturating with 66 % ethyl alcohol saturated with sodium chloride, and, after centrifuging, the supernatant solution was removed. This process was repeated until the supernatant fluid was clear, 3 to 4 washings being sufficient.

The glycogen was hydrolyzed in the same tube with 12 ml. of 2·2 % hydrochloric acid for 3 hours at 100° and, after cooling, the solution was neutralized with 40 % sodium hydroxide. It was rendered just acid with hydrochloric acid, filtered into a 25 ml. volumetric flask and, after rinsing the centrifuge tube and filter-paper with water, made up to volume. Dr G. A. Grant kindly estimated the fermentable sugar in the hydrolysate, employing the procedure of Harding & Grant [1933]. This eliminated all errors due to non-carbohydrate reducing substances which appear to be relatively high in kidney tissue.

Six determinations of the glycogen content of the kidney cortex of different animals yielded results equivalent to 9, 10, 11, 11, 26, 28 mg. glycogen per 100 g. fresh tissue. The right and left kidneys of the same animal contained equal amounts of glycogen.

Table II shows the glycogen, free phosphorus, total acid-soluble phosphorus and lactic acid contents of kidney cortex before and after incubation for 2 hours at 37·5°. It is clear that the low glycogen content of the cortical tissue is insufficient to account for more than a very small portion of the lactic acid formed in 2 hours at 37·5°.

The formation of lactic acid from several carbohydrates and the effect of arsenate on the process were investigated, with the hope that further information might be obtained regarding the precursor of lactic acid. Ashford [1933] showed that the rate of glucose breakdown by brain tissue was entirely unaffected by the

Table II. *Glycogen, free phosphorus, total acid-soluble phosphorus and lactic acid contents of kidney cortex before and after incubation for 2 hours at 37·5°.*

All values are given in mg. per 100 g. freshly chopped tissue.

	Free P	Total acid-soluble P	Lactic acid	Glycogen
Tissue plus 25% trichloroacetic acid "Initial value"	29	87	40	—
Tissue plus isotonic saline	65	98	127	—
Tissue plus absolute alcohol "Initial glycogen value"	—	—	—	9·0
Tissue plus isotonic saline "Final glycogen value"	—	—	—	0·24

presence of arsenate. On the other hand it has been known for many years that arsenate accelerates the rate of breakdown of hexosediphosphate by yeast preparations; moreover, recent investigations of Pett & Wynne [1934] have supported the suggestion that the stimulating effect of arsenate ions on the liberation of inorganic phosphate from hexosephosphate is exerted, not directly on the phosphatase system, but rather on the glycolytic system. The weighing bottles were set up as follows:

I. Tissue plus 5 ml. of the carbohydrate solution.

II. Tissue plus 5 ml. of the carbohydrate solution plus arsenate.

III. Tissue plus 5 ml. of isotonic saline.

Fresh solutions of Pfanstiehl monosaccharides were prepared on the morning of each experiment. From the barium salt of synthetic glucose-6-monophosphoric acid, kindly presented by Dr P. A. Levene, a solution of the sodium salt was prepared by treatment, at 80°, with the calculated amount of sodium sulphate. The mixture was placed in the refrigerator overnight and the next morning, just before using, was centrifuged for 35 min. The supernatant solution was carefully poured off and tested for freedom from barium ions. Barium hexosediphosphate, prepared from yeast, was ground in a mortar with the calculated amount of sulphuric acid in dilute solution; the mixture was centrifuged and the solution of the sodium salt was poured off and tested for freedom from barium ions. Before using, it was adjusted to *p*H 7·0 with solid disodium hydrogen phosphate.

The colorimetric method devised by Pett [1933] was employed for the estimation of inorganic phosphate and total acid-soluble phosphate in the presence of arsenate ions.

The results in Table III show that glycolysis in kidney tissue is very marked and is practically unaffected by the presence of *M*/500 arsenate. Fructose is much more slowly converted into lactic acid than is glucose. Kidney tissue is apparently unable to convert mannose and galactose into lactic acid, either in

Table III. *Percentage conversion of carbohydrates (0·1%) into lactic acid by kidney cortex in 3 hours at 37·5° in the presence and absence of 0·002* M *arsenate.*

Glucose	Glucose + arsenate	Fructose	Fructose + arsenate	Mannose	Mannose + arsenate	Galactose	Galactose + arsenate	Glycogen	Glycogen + arsenate
58	64	14	15	0	0	0	0	51	37
49	63	27	27	4	0	0	13·6	43	41
52	49	18	16	2	1	0	0	20	21
63	62	—	—	—	—	—	—	27	31
44	50	—	—	—	—	—	—	—	—
61	60	—	—	—	—	—	—	—	—

the absence or the presence of arsenate. Glycogen gives rise to lactic acid but the amount formed is smaller than that produced from glucose.

Other compounds tested were hexosediphosphate and glucose-6-monophosphate. The results with hexosediphosphate were somewhat variable but they suggest (Exps. 3 and 4, Table IV) that added hexosediphosphate can, at least partially, be transformed into lactic acid. Glucose-6-monophosphate gave variable results (Table V). The irregularity of the results with the hexosephosphates is difficult to explain; in no case was the amount of extra lactic acid formed as great as in the glucose experiments.

Table IV. *Percentage conversion of hexosediphosphate into lactic acid by kidney cortex in 3 hours at 37·5°.*

Hexosediphosphate added in each case was equivalent to 5 mg. lactic acid.

Experiment	1	2	3	4	5
% conversion	0	4	13	17	0

Table V. *Phosphate liberation and lactic acid production from glucose-6-monophosphate in the presence and absence of 0·002* M *arsenate.*

Phosphorus values are given in mg. per 100 g. freshly chopped tissue.

	Time in	Free P					Total acid soluble P					% conversion of added ester to lactic acid					
	hours	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	F
Tissue plus isotonic saline	2	69	65	73	88	83	93	113	113	131	123	—	—	—	—	—	—
Tissue plus 5 ml. hexosemonophosphate = 5 mg. lactic acid	2	84	80	89	104	93	125	122	144	164	146	0	7	45	5	4	41
Tissue plus 5 ml. hexosemonophosphate ≡ 5 mg. lactic acid plus arsenate Total concentration of arsenate, *M*/500	2	—	—		106	89	—	—	—	155	159	—	—	—	10	9	—

It is of interest to note that even when the tissue was incubated for 4 and 5 hours with hexosemonophosphate, no lactic acid in excess of the relatively small amounts produced during 2-hour incubation periods was observed.

The results, while variable, tend to substantiate the view that the greater portion of lactic acid produced by the cortical tissue of the kidney arises from glucose by some pathway independent of phosphate. It is not unlikely, however, that the small amount of glycogen in the kidney is broken down to lactic acid via hexosediphosphate.

On incubating cortical tissue with glucose and hexosediphosphate it was found that the latter did not appreciably increase the rate of lactic acid formation from glucose. Meyerhof [1918] showed that the induction period frequently observed on incubating maceration extract of yeast with sugar could be abolished by adding a small amount of hexosediphosphate. However, no such catalytic effect of this substance on the formation of lactic acid in either muscle or kidney has been observed.

The formation of lactic acid from pyruvic acid.

Sodium pyruvate solution (*p*H 7·0) in a concentration of 0·1 *M* and in the presence of rabbit kidney cortex was observed to give rise to appreciable amounts of lactic acid in excess of that formed in control experiments. As in the other experiments, no special precautions were taken to ensure anaerobic conditions. The results supplement those of Jost [1934] whose experiments were conducted anaerobically.

DISCUSSION.

The work of Jost [1934] suggests that under anaerobic conditions ox kidney brei converts carbohydrates into lactic acid by the same series of reactions as those postulated by Embden for skeletal muscle. Definite evidence of an alternative path was not obtained by Jost. The present experiments indicate that in rabbit kidney there may be an alternative system of glycolysis in which, at any rate under aerobic conditions, phosphate is not primarily involved. In support of this view are the following observations. (1) Lactic acid production and liberation of inorganic phosphate appear to be independent phenomena. (2) Hexosediphosphate and glucose-6-monophosphate fail to yield uniformly positive results with kidney whereas they are converted by muscle into lactic acid. (3) Arsenate does not appreciably accelerate the rate of lactic acid formation from carbohydrates by rabbit kidney. In view of the fact that Meyerhof [1927] and Meyerhof & Lohmann [1927] have shown that arsenate increases the rate of lactic acid formation, by fresh extracts of rabbit muscle, from glucose in the presence of hexokinase, from hexosediphosphate, from the Robison ester and from polysaccharides, the present results suggest that glycolysis in muscle and in kidney may normally proceed by different routes.

SUMMARY.

1. Sodium fluoride, in the concentration used, completely inhibits lactic acid formation in the excised kidney. No appreciable inhibitory effect is noted on phosphate liberation. The inference is that the two mechanisms are entirely independent.

2. The mechanisms controlling the liberation of phosphoric acid and the production of lactic acid are not significantly affected by $M/100$ cyanide.

3. Sodium iodoacetate inhibits lactic acid formation in kidney cortex. This drug has, however, no appreciable effect upon the liberation of inorganic phosphate. A loss of lactic acid is observed in the cortical tissue after incubation for 3 hours at 37·5° in the presence of iodoacetate.

4. The total glycogen content of the cortical tissue is not sufficient to account for the lactic acid formed when such tissue is incubated for 2 hours at 37·5°.

5. Kidney cortex produces large amounts of lactic acid from glucose, smaller amounts being formed from glycogen and even smaller quantities from fructose. Galactose and mannose give negative results. The results with hexose-mono- and diphosphates are variable.

6. Arsenate ions do not accelerate glycolysis in the cortical tissue of the kidney.

7. Pyruvic acid is readily reduced to lactic acid by kidney cortex and may, therefore, function as an intermediary in glycolysis.

The author wishes to express her gratitude to Profs. H. Wasteneys and A. M. Wynne of this department and to Dr E. T. Waters of the Department of Physiology for their continual interest and advice in this work.

Many thanks are due to the Banting Research Foundation for a personal grant and for one which in part defrayed the cost of the experiments.

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CCLXXX. TECHNIQUE OF THE LINE TEST ASSAY FOR VITAMIN D.

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(*Received 10 September 1936.*)

Coward & Key [1934] have reported the successful use of a modified technique in the now well-known "line" test for the assay of vitamin D. In this test animals of suitable age and pre-experimental history are given a rachitogenic diet for about 3 weeks. They are then divided into groups, the sexes and litter-mates being distributed as uniformly as is necessary to give trustworthy results, and the animals in each group receive a measured dose of the substance under test or of a standardized preparation of the vitamin. Coward & Key, and other workers, had previously divided these doses into equal portions, one for each day of the test period, which is usually 10 days. In the paper cited, the authors report that, in a direct comparison between litter-mates, they obtained identical results, of equal accuracy, whether they gave to the animals a certain amount of the vitamin either as a tenth of the total dose daily throughout the 10 days' test period or as one dose at the beginning of that period.

Obviously such a modification of technique presents advantages, mainly in economy of time, and it seemed to us necessary to discover whether it could be adopted in the somewhat different circumstances of our own laboratory. For reasons published elsewhere [Bacharach, 1936] we find it necessary to use a much more severely rachitogenic diet than the 2965 of Steenbock & Black [1925], which was used by Coward & Key. Another, but a minor, difference is that we examine the femora instead of the ulnae and radii.

In the first instance two litters of animals were used, and approximately half the animals in each litter were dosed with a solution of previously assayed irradiated ergosterol diluted so that 1 drop contained 0·5 International Unit (I.U.) of vitamin D. Each of these animals was given 1 drop daily for 10 days. The other half of the animals each received on the first day of the test a single drop of a solution containing the same irradiated ergosterol at ten times the concentration of the first solution. The degrees of healing found in the stained bones are shown in Table I.

Table I.

Dose	Individual healing	Average healing
Single	0, 0, ½, ½, ½, ½, ½, 1	0·4
Divided	1, 1, 1, 1, 1, 1, 1½	1·1
Negative control	0, 0	0

It was thought possible that the single dose method might, through incomplete consumption of a single drop by the animals, have involved a purely mechanical error of unduly high effect. A further test was therefore made, using a dilution of the International Standard Preparation (VD 10) such that 1 drop contained approximately 0·25 I.U. of vitamin D. This was fed at two levels, both by the single and the divided dose methods; the single dose was given as 10 or 20 drops on the first day of test, the divided dose as 1 drop or 2 drops on each day (Table II).

Table II.

Dose		Individual healing	Average healing
0·25 unit	Single	0, 0, 0, 0, 0	0
	Divided	0, 0, 2, 1	0·75
0·5 unit	Single	0, 0, 0, ⊙, ½, 1	0·25
	Divided	0, ⊙, ½, 1, 1	0·6
Negative control		0, 0	0

⊙ indicates only one or two spots of calcified tissue.

Here one litter was used for the 0·25 unit and the other for the 0·5 unit dose, so that the degrees of healing on the two doses were not necessarily comparable. Again, however, we seemed to have evidence that the divided dose method gives greater healing.

The final experiment involved the use of 8 litters, including 64 animals. An intermediate course was adopted between those taken in the preliminary experiments, as it was considered undesirable to administer as much as 20, or even 10, drops (each 22·5 mg.) of oil solution on one day to an animal.

Accordingly one test solution contained 0·005γ of calciferol per drop; 3 drops were given daily, so that the daily dose was 0·6 I.U., and the total experimental dose 6 units. This solution also contained sufficient β-carotene[1] to give every 3 drops a vitamin A equivalence of 5 I.U.

The second solution contained 0·0375γ of calciferol per drop, of which 4 were given on the first day of the test period. Thus the total dose was also 0·15γ of calciferol, or 6 I.U. Carotene[1] equivalent to 50 I.U. was administered separately during the first 2 days of the test period (Table III).

Table III.

Dose	Number of animals	Range of healing	Average healing
Single	28	⊙ to 2	0·91
Divided	27	1 to 2	1·41
Negative control	9	0 to ½	0·11

An examination was also made of the differences between males and females (Table IV).

Table IV. *Average healings.*

	Males	Females	Average of males and females
Single dose	1·07 (15)	0·73 (13)	0·90
Divided	1·47 (17)	1·30 (10)	1·39

Numbers in brackets represent number of animals.

The averages shown in Tables III and IV are all calculated from the appropriate bone-scores, irrespective of the number of animals in any given litter that may be receiving a particular dose. Table V has been drawn up by calculating the means of the litter means for each dose, taking the sexes separately or together.

Table V.

		Average of healing by litters		
	Average of both sexes	Males	Females	Males and females
Single	0·93	1·20	0·76	0·98
Divided dose	1·41	1·41	1·33	1·37

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[1] The feeding of carotene in this experiment had no bearing on the problem under examination and was practised for reasons not germane to it.

DISCUSSION.

It seems clear that, even under the best conditions, the technique recommended by Coward & Key cannot be applied to animals of our particular stock and upbringing. Whether some limitation to the maximum possible daily absorption or utilization of vitamin D, or to both, is imposed by the intensely rachitogenic nature of our diet 401, or whether some unsuspected difference operates, we are unable to decide.

There are indications that males respond somewhat better to cure by a given dose, that is, are less rachitic than females. This is contrary to the view of Morgan [1932] but it is not inconsistent with the slight superiority in bone-ash content of females over that of males of approximately the same age [Bacharach, 1936]; the males have less distance to go to achieve normal calcification. Slight differences in result can be obtained by various methods of calculating the average degrees of healing, but these differences seem to become unimportant when as many as eight different litters are used and over 25 animals receive any one dose, even though the sex distribution be not perfectly symmetrical. Provided that a grand average is finally taken, the results are little affected. In the present instance the single and the divided dose healings showed figures of

0·91 to 1·41,
0·90 to 1·39,
0·93 to 1·41,
0·98 to 1·37,

according to the method of averaging.

We have no evidence to show whether, with our animals and under our experimental conditions, the two methods of administering vitamin D supplements, if used independently, would give the same accuracy of result. But it seems clear to us that, with those animals under those conditions, it is not justifiable to compare one source of vitamin D with another unless both are administered in distributed doses. It is possible that it might be satisfactory to give both sources by the single dose method, but of this we have no direct proof.

SUMMARY.

The single dose method of feeding vitamin D supplements in the line test, as advocated by Coward & Key, was found not to be applicable to a different stock of rats receiving a more severely rachitogenic diet and gave significantly less good healing than the divided dose method.

Males appear to be slightly less rachitic than females under similar conditions of slowly healing rickets.

We take this opportunity of acknowledging the kindness of Dr K. H. Coward, who read the manuscript of this paper before it was submitted for publication and made certain suggestions for modification, all of which have been adopted.

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CCLXXXI. THE ISOLATION OF METHYLMALONIC ACID FROM RAT URINE.

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(*Received 21 September 1936.*)

In isolating the metabolic products of anthracene from rat urine considerable difficulty was encountered in separating the glycuronic acid in a pure condition. Large volumes of acidified urine from rats fed as previously described [Boyland & Levi, 1935] were extracted with ether for 72 hours. The glycuronic acid was extracted by the ether but on account of its low solubility in ether crystallized out in the extract. The ether appeared to contain other acids but very little glycuronic acid. The ethereal extracts were concentrated to a semicrystalline syrup which was drained on a porous plate. The major part of the solid was found to be crystallizable from hot toluene and proved to be methylmalonic acid. Between 200 and 500 mg. of the acid were obtained from each litre of urine. The acid crystallized in plates, M.P. 126–128°. It was soluble in water, alcohol and acetone, and the solutions were optically inactive. It did not react with HNO_3, Br_2 or $KMnO_4$. Heated in a dry tube it gave an oily acid distillate. It crystallized from water in the form of a hydrate containing 13·24 % H_2O. $C_4H_6O_4, H_2O$ requires 13·22 % H_2O. (Analysis of anhydrous material from toluene (Weiler). Found: C, 40·9; H, 5·00 %. $C_4H_6O_4$ requires C, 40·8; H, 5·08 %. Neutralization equivalent, 61. Theoretical for dibasic acid, 59.)

0·5 g. of the acid was heated in a tube and the distillate collected in an aqueous suspension of Ag_2O. The silver oxide suspension was washed and excess Ag_2O removed by filtration at 100°. The filtrate on cooling gave crystals of a silver salt which were collected and dried. A weighed amount of this was ignited and gave 59·2 % Ag. The theoretical value for silver propionate is 59·3 % Ag.

The acid isolated has all the properties of methylmalonic acid, the M.P. of which is stated in the literature to be between 120° and 130° according to different authors. The formation of a monohydrate by this acid does not appear to have been previously described.

More rats were fed on exactly the same diet as previously described except that no anthracene was present. The urine was collected, acidified, filtered and extracted with ether for 24 hours. The ethereal extract from 4 litres urine was boiled with charcoal, filtered and evaporated to 20 ml. The crystals which separated on keeping the residue in a desiccator were filtered off and dried; yield 0·4 g. Part of the crude material was crystallized from hot toluene and then melted at 124–126° and gave propionic acid on heating to 150°.

It was found impossible to isolate methylmalonic acid from rabbit urine or from human urine. In one experiment the combined ethereal extract of 34 litres of male human urine was treated in a manner similar to that described above.

The precursor of the methylmalonic acid is as yet unknown, but it could conceivably arise from thymine, the side chain of cholesterol, carotenoids or the methylpyrrole of haemoglobin and other pigments. Of these, however, the simplest precursor is thymine which on oxidation and hydrolysis would give urea and methylmalonic acid. In the breakdown of thymonucleic acid, if all the purines were converted into uric acid and all the thymine into methylmalonic acid, there should be one molecule of the latter to two molecules of uric acid.

Szent Györgyi has shown that sodium malonate causes inhibition of respiration and of acetoacetate breakdown in liver slices, but we have found that sodium methylmalonate does not have this effect.

SUMMARY.

Methylmalonic acid has been isolated from rat urine where it may occur to the extent of 0·5 %.

One of us (A. A. L.) has pleasure in thanking the Sir Halley Stewart Trust for a fellowship held during the progress of this work.

REFERENCE.

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CCLXXXII. THE ACCURACY OF VITAMIN A DETERMINATIONS. THE STARTING-POINT OF THE TEST PERIOD.

BY KATHARINE HOPE COWARD.

From the College of the Pharmaceutical Society, London.

(*Received 30 September 1936.*)

MOST vitamin A tests in this country are made by the "increase in weight" method; i.e. young rats which have become steady in weight on a diet deficient in vitamin A are given doses of the test substance or standard for a specified period. The resultant increases in weight are used to determine the potency of the test substance in terms of the standard of reference.

Some discussion has arisen in the last few years as to the best point of each animal's weight curve to consider as the beginning of the "increase in weight". Coward and co-workers have always used the point of the curve at which the animal was considered steady in weight and given the first dose of vitamin A. The test period, i.e. the period during which doses of vitamin A were given, was then always of the same length (5 weeks in early work, reduced later to 3 weeks). Baumann *et al.* [1934] obtained good results by counting the point at which the rat's weight passed the highest point reached in the preparatory period as the beginning of the test period and ignoring the part of the rat's curve where it was regaining this weight. Eddy [1936], however, decided that the method of calculating the 28 days' gain had little influence on the accuracy of the test. Various other workers have suggested (verbal communications) that this method of counting the period of the test might give more accurate results than counting it from the first day of dosing. In this method the time during which doses were given to some of the rats would have to be prolonged beyond the set period of the test. Some rats might never (within the limits of the patience of the worker) reach the needed starting-point for the test and they would have to be discarded.

It seems possible to test this suggestion for increasing the accuracy of a determination by the following consideration. The recovery, if any, to the suggested starting-point would generally take place within the first 2 weeks of dosing. Therefore, the accuracy obtainable in the last 3 weeks (say) of a 5 weeks' test, should be greater than that obtainable in the first 3 weeks of the test which included all the responses of the type described. A calculation has been made of the accuracy of the test obtainable during each of the 5 weeks of dosing. This was based on the calculation of $\lambda=\sigma$/the slope of the line relating increase in weight to $\log_{10}$ of dose given. Coward [1933] showed that the relation between the mean variance of the increase in weight of rats and the duration of the test period was a straight line for the whole of the 5 weeks for male rats, and also for the first 3 weeks for female rats, though the curve for these flattened a little in the fourth and fifth weeks. Thus the variance increased by an equal amount during each week of the test period. Therefore the variance in the increase in weight during the first, second, third, fourth and fifth weeks was the same for the male rats, and for the female rats slightly less in the fourth and fifth weeks than in the first 3 weeks. Hence the standard deviation of the increase in weight during the first, second, third, fourth and fifth weeks was the same for the male rats, and for the female rats slightly less in the fourth and fifth weeks.

The curves for the first week have already been published [Coward, 1933]. It remained then to determine the curves of response to doses of vitamin A during the second, third, fourth and fifth weeks, both for the male and female rats. This was done by the use of the formula

$$\tan(\alpha-\beta)=\frac{\tan\alpha-\tan\beta}{1+\tan\alpha\tan\beta},$$

where, for example,

$\tan\alpha$ was the slope (b_1) of the curve of response for a 2 weeks' test;

$\tan\beta$ was the slope (b_2) of the curve of response for a 1 week's test;

$\tan(\alpha-\beta)$ was the slope (b) of the curve of response for the increase in weight during the second week.

The values of b (the slopes of the curves) in the equation had to be divided by 100 to reduce the unitage of y to that of the abscissae. The slopes of the curves of response to doses in the first week, and in the second, third, fourth and fifth weeks found by this method may be seen in Table I.

Table I. *The slopes of the curves of response relating increase in weight during the first, second, third, fourth and fifth weeks respectively to the log of the dose of vitamin A given.*

	Male rats	Female rats
1st week	9·05	6·64
2nd week	10·05	6·65
3rd week	9·78	4·37
4th week	7·59	4·37
5th week	6·59	4·26

It is evident that with the exception of the first week's response of the male rats and perhaps the third week's response of the female rats, the two sets of curves fall into two nicely graded series. The inaccuracy of the test may be measured by

$$\lambda=\frac{\sigma}{\text{slope of curve of response}},$$

and since σ is constant, then the greater the slope of the curve of response, the less is the inaccuracy of the test. The smaller value of σ in the fourth and fifth week's variance of the response of the does lowers the values for λ very little. Therefore the conclusion must be drawn that each successive week's dosing gives a slightly less accurate result than that of the week preceding it. It would, therefore, seem to be preferable to treat the responses to doses of vitamin A in the simplest way, i.e. to count the period of the test from the day on which the first dose is given. This prevents the prolongation of a test for the sake of a few rats, which is an economy of time and which also prevents the possibility of the results from these same rats being influenced by changes in conditions which would not affect the results from periods already completed.

This calculation was based on figures obtained from 960 male rats and 1110 female rats.

SUMMARY.

Different workers have suggested that the recovery period in a vitamin A determination should be taken as starting on that day on which the rat receiving doses of vitamin A exceeds the highest weight it had attained during the preparatory period when it was not receiving any vitamin A.

This suggestion has been tested on a colony of rats in which all the animals lost weight (some of them only a few g.) before they were given doses of vitamin A, and many of them continued to lose weight for a short time before

recovery began. With most animals the point suggested above as the starting-point for the calculation of increase in weight was attained within 1 or 2 weeks (if ever) from the day on which vitamin A was first given. Hence a calculation of the accuracy of the results obtained during successive weeks should indicate whether it is desirable or not to ignore the period previous to the point suggested as the starting-point for the calculation.

The accuracy obtainable during each week of a 5 weeks' test period is determined from $\lambda=\sigma/\text{slope}$ of curve of response. It has been shown that since σ is the same for successive weeks and the slope of the curve of response for each week's increase in weight becomes slightly less in successive weeks, the accuracy of the result of each week's dosing is slightly less than that of the preceding week. Hence the greatest accuracy is obtained by counting the test period from the first day of dosing.

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CCLXXXIII. THE INFLUENCE OF THE LENGTH OF THE TEST PERIOD ON THE ACCURACY OBTAINABLE IN A VITAMIN B_1 TEST.

By KATHARINE HOPE COWARD.

From the College of the Pharmaceutical Society, London.

(*Received 30 September 1936.*)

It was shown by Coward [1933] that the accuracy obtainable in a vitamin A test by the "increase in weight" method was increased very little by prolonging the test period beyond three weeks. Since then, enough results of vitamin B_1 determinations by the method of the "increase in weight" of rats have been accumulated to make it possible to carry out a similar examination of the duration of a vitamin B_1 test.

The technique of the test has been described by Coward *et al.* [1933]. Briefly, it consists of measuring the increase in weight of rats which, having become steady in weight on a diet deficient in vitamin B_1, are then given doses of (*a*) the International Standard (I.S.) for vitamin B_1 or (*b*) the substance under examination. With the description of the test was published an estimate of the accuracy obtainable. It was much greater than that obtainable in a vitamin A test of the same length of time. Thus a smaller number of animals would be required for a vitamin B_1 test for a given degree of accuracy than would be required for a vitamin A test of similar accuracy.

In the determination of the vitamin B_1 content of a substance we have generally divided the rats into 4 or 5 groups, two of the groups being given doses of 0·01 and 0·02 g. respectively of the I.S. and two of the groups doses in the ratio of 1 : 2 of the substance under examination or, if no information on the possible potency of the substance was available, the doses chosen for a preliminary test were in the ratio of 1 : 3 or 1 : 5. A group of rats given no dose was included when it was desired to show whether even a large dose of the test substance was or was not better than no dose at all. The different groups have generally contained equal numbers of bucks and does but it was shown in the paper by Coward *et al.* [1933] that the curve of response of bucks was very little steeper than that of does; hence it was not really necessary to have equal numbers of each in each group. By this means we have obtained a large amount of information not only on the variation in response to doses of vitamin B_1 but also on the variation in the slopes of the curve of response. It has confirmed our previous estimate of the high degree of accuracy attainable and it has shown that the accuracy obtainable by carrying on the test for three weeks is only very slightly greater than that obtainable by carrying it on for two weeks.

The average variance (σ^2) of the increase in weight in 1, 2 and 3 weeks.

The average variance of the response of male and female rats to doses of vitamin B_1 was calculated by the formula usually employed:

$$\sigma^2 = \frac{\Sigma d^2}{N - M},$$

where Σd^2 is the sum of the squares of all the deviations from their respective means; N is the number of rats from which the calculation was made; M is the number of groups of rats in which the N rats were distributed.

The results for male and female rats for 1, 2 and 3 weeks' tests are collected in Table I.

Table I. *The average variance and standard deviation of male and female rats to doses of vitamin B_1 in 1, 2 and 3 weeks' tests.*

		Σd^2	N	M	σ^2	σ
Male rats	1 week's test	4132·0	306	110	21·08	4·31
	2 weeks' test	8512·5	295	105	44·80	6·69
	3 ,,	12050·5	282	101	66·58	8·16
Female rats	1 week's test	3660·5	243	96	24·90	4·99
	2 weeks' test	5628·0	229	92	41·08	6·41
	3 ,,	7173·0	215	85	55·18	7·43

The average variance of response in 1, 2 and 3 weeks when plotted against the time gives a nearly straight line relationship for both the male and female rats. The line for the male rats has a greater slope than that for the female rats, but the two lines intersect. That for the female rats does not pass through or near the origin. (Compare with the slopes of the lines relating average variance in response to doses of vitamin A with the duration of the test [Coward, 1933].)

The standard error of the average result from 10 animals is $\frac{\sigma}{\sqrt{10}}$, the probable error is $\frac{2}{3}\cdot\frac{\sigma}{\sqrt{10}}$ and the probable error of the difference between the average responses of two groups of rats (e.g. one given a dose of the standard and one given a dose of test substance) is $\frac{2}{3}\cdot\frac{\sigma}{\sqrt{10}}\cdot\sqrt{2}$.

The curves of response of male and female rats to doses of vitamin B_1 in 1, 2 and 3 weeks.

The difference in the average responses to all the pairs of doses in the ratio 1 : 2 of all substances tested were calculated for the 1, 2 and 3 weeks' tests. (The response of any animal that died during the 3 weeks was used for the 1 or 2 weeks previous to death. No result from it was counted in the week of its death.) The weighted mean of these differences was determined. This was permissible since it had been shown by Coward *et al.* [1932] that the line relating increase in weight in 3 weeks to dose of vitamin B_1 given is logarithmic. This average value is the difference in increase in weight due to a doubling of the dose of vitamin B_1 given. Therefore, in the curve of response relating increase in weight to the logarithm

Table II. *The calculation of the slopes of the curves relating increase in weight in 1, 2 and 3 weeks to the logarithm (to the base 10) of the dose of vitamin B_1 given.*

		Average difference between increases in weight from doses in ratio 1 : 2	Slope of curve of response (Diff./log 2)
Male rats	1 week's curve	6·23	20·70
	2 weeks' curve	11·09	36·88
	3 ,,	15·88	52·76
Female rats	1 week's curve	5·28	17·54
	2 weeks' curve	11·94	39·67
	3 ,,	16·86	56·01

of the dose of vitamin B_1 given, the interval for the average value of y corresponds to an interval of 0·30103 ($\log_{10} 2$) for x. The slopes of the curves of response are then obtained by dividing the interval (average differences in the increase) by 0·30103 (Table II).

There is not as great a difference between the slopes of the curves relating increase in weight of male and female rats to doses of vitamin B_1 as there is to doses of vitamin A or Vitamin D [Coward, 1933; Coward *et al.*, 1932].

The determination of the accuracy obtainable in 1, 2 and 3 weeks' tests.

The accuracy obtainable by carrying on the test for 1, 2 or 3 weeks is determined from the formula $\lambda = \sigma/\text{slope}$ of curve of response (Table III). The probable error of a determination in which 20 animals were used, ten of them being given a dose of the standard and the other ten a dose of test substance is determined from the formula

$$\frac{2}{3}\cdot\frac{\sigma}{\sqrt{10}}\cdot\frac{\sqrt{2}}{\text{slope}} \text{ or } \frac{2}{3}\cdot\frac{\lambda}{\sqrt{10}}\cdot\sqrt{2}.$$

Table III. *The accuracy obtainable by carrying on the test for 1, 2 or 3 weeks with 10 pairs of animals, one of each pair having been given a dose of the standard and the other a dose of test substance.*

		σ	Slope of curve	$\lambda = \sigma/\text{slope}$	$\frac{2}{3}\cdot\frac{\lambda}{\sqrt{10}}\cdot\sqrt{2}$	Antilog range of dose ratio	Range of probable error of determination %
Male rats	1 week's test	4·31	20·70	0·208	0·0620 $\bar{1}$·9380	1·153 0·867	+ 15·3 − 13·3
	2 weeks' test	6·69	36·88	0·181	0·0539 $\bar{1}$·9461	1·132 0·883	+ 13·2 − 11·7
	3 ,,	8·16	52·76	0·155	0·0462 $\bar{1}$·9538	1·112 0·899	+ 11·2 − 10·1
Female rats	1 week's test	4·99	17·54	0·284	0·0846 $\bar{1}$·9154	1·215 0·823	+ 21·5 − 17·7
	2 weeks' test	6·41	39·67	0·162	0·0483 $\bar{1}$·9517	1·118 0·895	+ 11·8 − 10·5
	3 ,,	7·43	56·01	0·133	0·0396 $\bar{1}$·9604	1·096 0·913	+ 9·6 − 8·7

Thus the probable error of a determination of vitamin B_1 in which ten rats are used for the standard and ten for the substance whose potency is being determined is less in a 2 weeks' test than in a 1 week's test, and only slightly less in a 3 weeks' test than in a 2 weeks' test. Indeed the increase in accuracy gained by carrying on a test for a third week cannot be considered worth the extra labour involved. With equal numbers of male and female rats on standard and test substance, the ratio of the accuracies of 1, 2 and 3 weeks' tests is about 18 : 12·5 : 10 %.

The number of animals on which this calculation was based was 549 for the 1 week's test, 524 for the 2 weeks' test and 497 for the 3 weeks' test, some of the rats on the low doses having died during the course of the experiments.

SUMMARY.

The average variance in response of rats to doses of vitamin B_1 has been determined for tests of 1, 2 and 3 weeks' duration.

The slopes of curves of response relating increase in weight in 1, 2 and 3 weeks and dose of vitamin B_1 given have been constructed by averaging the differences in response obtained by giving two doses in the ratio 1 : 2 of numerous substances. This method is justified by its having been shown in a previous paper that the relation between increase in weight and dose of vitamin B_1 given is logarithmic.

The accuracy obtainable in the test when carried on for 1, 2 and 3 weeks has been determined by the formula $\lambda = \sigma/$slope of the curve of response. The probable errors of determinations of the vitamin B_1 potency of a substance by comparison with the standard tested simultaneously, when 10 animals are used on each substance, and when the tests are carried on for 1, 2 and 3 weeks, are about 18, 12·5 and 10% respectively. Thus the increased accuracy obtained by carrying on a test for longer than 2 weeks is seldom worth the extra labour involved.

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CCLXXXIV. THE ULTRAVIOLET SPECTRUM OF HAEMOGLOBIN AND ITS DERIVATIVES.

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Since Soret [1883] described an ultraviolet absorption band in three of the haemoglobin series, numerous investigations have been carried out on the ultraviolet absorption spectrum of the blood pigment and its immediate derivatives. The haemoglobin compounds were investigated by Gamgee [1895] who pointed out that there was a specific ultraviolet band common to oxyhaemoglobin, reduced haemoglobin, carbon monoxide haemoglobin, nitrous oxide haemoglobin, haemochromogens, haematin hydrochloride, methaemoglobin and haematoporphyrin which was absent from the spectra of bilirubin, hydrobilirubin and urobilin. Kobert [1900] investigated several haemoglobin compounds and demonstrated the existence of a band in the ultraviolet which was common to the spectra of oxyhaemoglobin, carbon monoxide haemoglobin, alkaline methaemoglobin and cyanide haemoglobin and concluded that there was a structure common to these compounds which possessed this band.

Subsequent investigations have fixed the position of the band as follows:

Investigator	Position of the ultraviolet band (Å.)
Rost *et al.* [1909]	4100
Lewin *et al.* [1907]	4150
Newcomer [1919]	4140
Schumm [1921]	4135–4140
Suhrmann & Kollath [1927]	4150
Hicks & Holden [1929]	4115
Berg & Schwarzacher [1930]	4134
Davis & Sheard [1934]	4180

Friedli [1926] investigated mesoporphyrin and haematoporphyrin and located a band common to both at 4000 Å. Triebs [1932] has made extensive investigations concerning spectra of fourteen porphyrins, including some made from haem, and has established the position of a common band at 4000 Å. Studies on bile and purified bile pigment yield a spectrum different from that of the haem and porphyrin pigments. Mann *et al.* [1926] studied the bile pigment, and found maximum absorption in the region of 4400 Å. Davis & Sheard [1934] published ultraviolet spectrum data of bile pigment in pure state. Their curve shows a heavy absorption from 5000 to 3600 Å. with a maximum at 4500–4600 Å.

The following investigations were undertaken in order to correlate the ultraviolet absorption spectra of haemoglobin compounds and derivatives with their chemical structures. These included a study of a wide variety of haemoglobin derivatives. Part of this work had been done previously, but the separate entities had been examined by different investigators with different apparatus and methods. In no instance could reference be found to a study of this kind having been carried out on the haemoglobin of a single species by one investigator and the same technique.

Methods. The instrument employed was a Bellingham and Stanley quartz spectrograph. The ultraviolet spectrum from 2100 to 5000 Å. was extended over 18 cm. upon the negative. The absorption measurements were determined by the use of a photoelectric recording photometer developed in this laboratory by Macallum & Coates [1933].

Recrystallized horse haemoglobin was used for the experiments. It was prepared by the usual methods for obtaining a purified haemoglobin solution and crystallized in an electrodialysis apparatus after a 4-hour period dialysis. It was recrystallized in the dialyser twice from a solution of cold water containing a small amount of bicarbonate. The crystals prepared in this way were dried in air and kept for periods of weeks without showing appreciable change to methaemoglobin.

The pure haem was prepared from solutions of the purified haemoglobin by a modification of the Schalfejeff method. It was recrystallized twice by dissolving it in chloroform solution containing a small amount of pyridine and allowing it to reprecipitate slowly after the cautious addition of acid. The recrystallization was repeated twice and the crystals thoroughly dried.

Crystalline haematoporphyrin was prepared from the recrystallized haemin by dissolving it in glacial acetic acid and saturating the solution with dry hydrogen bromide gas at 10°. It required 3–4 days to dissolve 5 g. of haemin in 50 ml. of glacial acetic acid by this method. Reprecipitation of the haematoporphyrin was brought about by the addition of sodium acetate. The ferric oxide was removed by sodium hydroxide. The haematoporphyrin was repeatedly reprecipitated with acid and finally crystallized *in vacuo* over sulphuric acid. Recrystallization was made from 10% hydrochloric acid and the crystals were "air dried".

The oxyhaemoglobin solutions were prepared from the dried horse haemoglobin solutions by weighing out the required amount to give a 0·2% solution, and dissolving it in distilled water with sufficient shaking to aerate the mixture and convert all the haemoglobin into oxyhaemoglobin. The spectrograms were taken at once.

The reduced haemoglobin solutions were made in the same way from the dried crystalline haemoglobin; to each 100 ml. of these solutions there were added 10 ml. of 10% sodium hydrosulphite in 5% ammonia in order to bring about complete reduction of all the haemoglobin. The hand spectroscope was used to detect the complete transformation of oxy- to reduced haemoglobin.

Neutral methaemoglobin was prepared by adding 10 drops of 10% potassium ferricyanide to 100 ml. of 0·2% oxyhaemoglobin. Alkaline methaemoglobin was similarly prepared except that the 0·2% oxyhaemoglobin had been prepared in 0·01 *N* ammonia. The degree of the change from oxyhaemoglobin to methaemoglobin was measured by the hand spectroscope.

The haemoglobin was converted into carboxyhaemoglobin by pure carbon monoxide.

The stock alkaline haematin was prepared by dissolving 13 mg. of dried haemin in 25 ml. of 0·5 *N* NaOH. For spectrographic purposes 0·25 ml. of the stock solution was diluted with 4·75 ml. of water. An alcoholic solution of alkaline haematin was made from the stock solution by adding 0·25 ml. to 4·75 ml. of absolute alcohol. The stock solution of acid haematin was made up by dissolving 13 mg. of the dried haemin in 10·0 ml. of 0·5 *N* NaOH. 0·5% gum arabic was added to this solution as a protective colloid in order to prevent precipitation of the pigment when 1·0 ml. of concentrated hydrochloric acid was added. 0·3 ml. of this stock was diluted to 5·0 ml. for spectrographic purposes. Acid haematin in alcohol solution was made by adding 0·3 ml. of the stock solution to 4·7 ml. of absolute alcohol.

Pyridine haemochromogen was made by adding small amounts of pyridine and sodium hydrosulphite to alkaline haematin solution. Piperidine haemochromogen was similarly prepared. A preparation of alkaline haematin and pyridine without reduction was also made and the spectrogram taken.

The bilirubin obtained for this work was the preparation used for intravenous injection and of a fairly high purity. It was purified further and crystallized from chloroform. It was dissolved in 0·01 *N* ammonia for spectrographic work. A solution was also made in a 50:50 alcohol-acetone mixture to which a small amount of 0·1 *N* NaOH had been added.

The globin was prepared from horse blood by the method of Hill & Holden [1926]. For spectrographic purposes a 10% solution was made. To prepare a denatured globin solution, the native protein was heated with 0·5 N hydrochloric acid for half an hour at 60°.

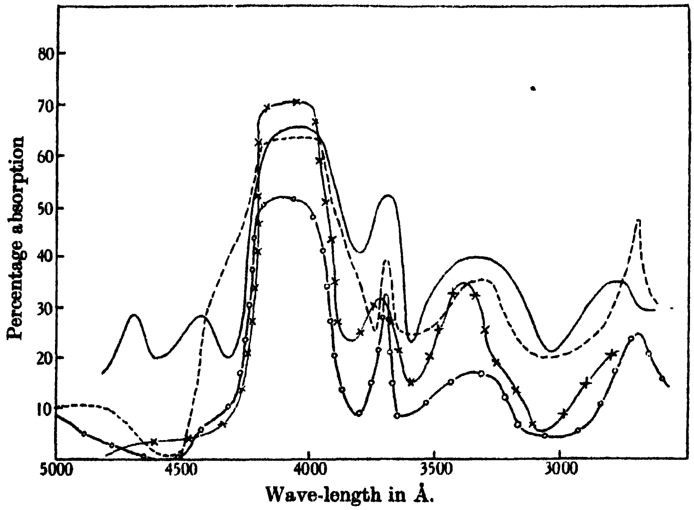

Fig. 1. x — x Alkaline methaemoglobin. ——— Neutral methaemoglobin. - - - - Oxyhaemoglobin. o—o Carboxyhaemoglobin.

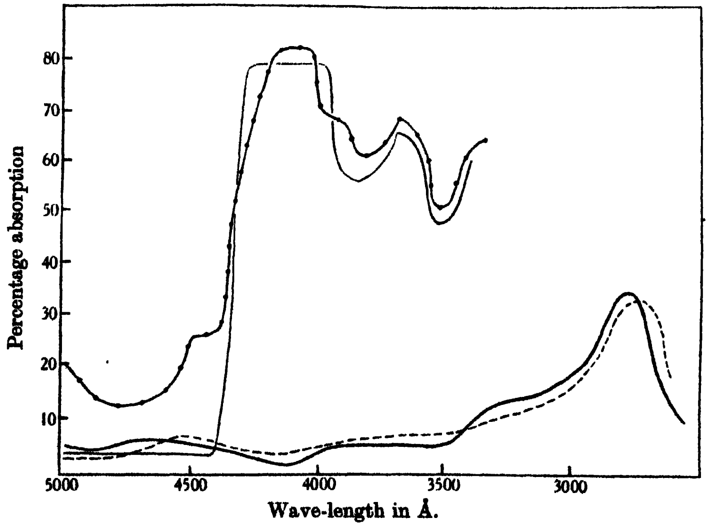

Fig. 2. ——— Native globin. - - - - Denatured globin. •—• Haemoglobin reduced in a tonometer. ——— Haemoglobin reduced by hydrosulphite.

Observations. Oxyhaemoglobin, reduced haemoglobin and carboxyhaemoglobin all give spectrum curves which are quite similar. All are characterized by the intense main band in the region of 4100 Å. while lesser absorption is shown at 3700 Å. There is another minor band at 3400 Å. which appears to be characteristic of the haemoglobin molecule. There is also a band at 2750 Å. Methaemoglobin in neutral and alkaline solution shows the typical absorption at 4100 Å. and the

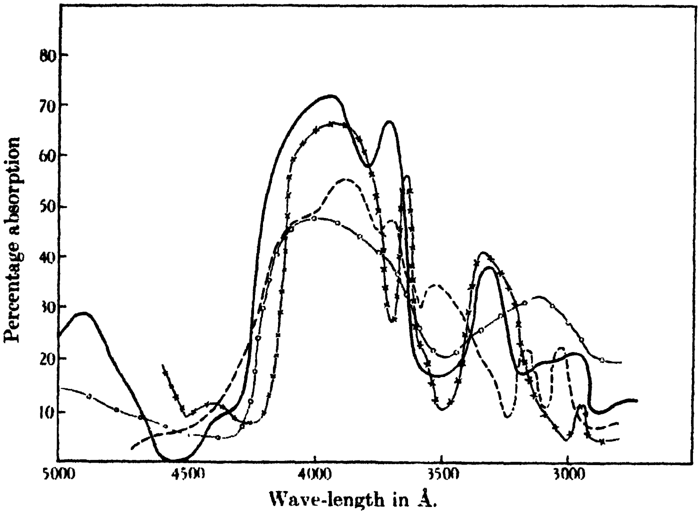

Fig. 3. ——— Acid haematin in alcohol. - - - - Acid haematin in water.
× — × Alkaline haematin in alcohol. o—o Alkaline haematin in water.

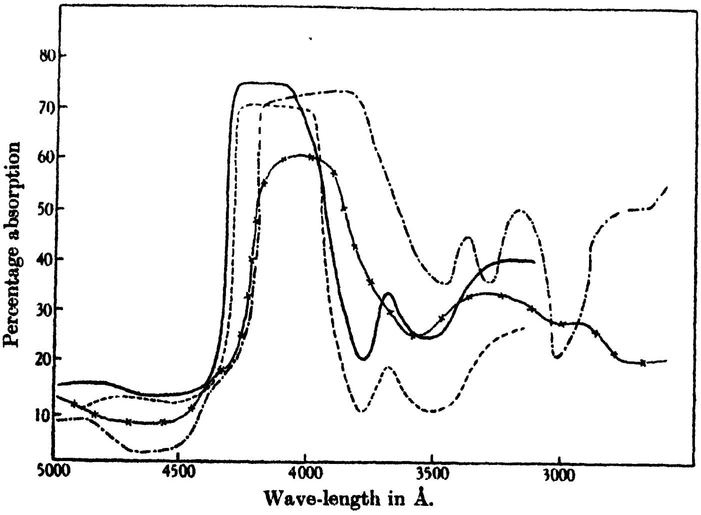

Fig. 4. —·—·—·— Haematoporphyrin. ——— Pyridine haemochromogen.
- - - - Piperidine haemochromogen. × — × Alkaline haematin (reduced).

haematin solutions show the same general type of curve. The haemochromogens have a very distinct band at 4100 Å. and a heavy general absorption below 3400 Å. The haematoporphyrin has a similar spectrum but the general absorption is greater. The bilirubin spectrum is entirely different. The absorption is very high from 5000 to about 4000 Å., while there are no areas of selective absorption. The globin, both native and denatured, gives a band at 2750 Å.

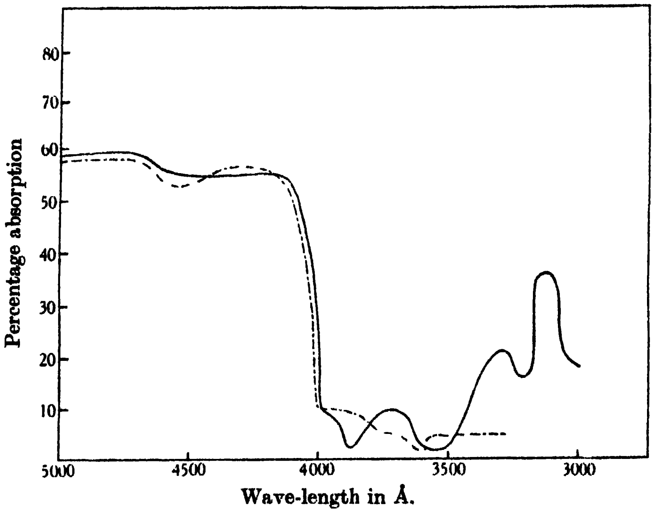

Fig. 5. ——— Bilirubin in alcohol-acetone solution. – – – Bilirubin in dilute ammonia.

DISCUSSION.

All the haemoglobin compounds and derivatives display a similar selective absorption in the ultraviolet. The "great" absorption band at 4100 Å. is found in the same general form in oxy-, reduced, carboxy-, and met-haemoglobin. The exact centre of the band is difficult to locate, since its determination depends on the selection of the boundaries of the band. The bands of oxyhaemoglobin and CO-haemoglobin are very similar, oxyhaemoglobin showing more general absorption. All have a small peak at 2780 Å. and a rather poorly defined band centred at 3400 Å. At 2700 Å. there is another band which has been shown to be due to the globin fraction [Lewis, 1917]. The curves for purified globin, both native and denatured, show this characteristic band in the region of 2700 Å. Methaemoglobin in alkaline solution has the same general spectrum as oxyhaemoglobin and CO-haemoglobin. In neutral solution it exhibits greater general absorption but the same type of curve. Reduced haemoglobin has the same general form except for increased general absorption, due to the presence of sodium hydrosulphite. This reagent has such a high absorption in the ultraviolet that a cut-off occurs about 3300 Å. The reduction of the oxyhaemoglobin by shaking *in vacuo* in a tonometer was also tried as a means of making reduced haemoglobin, but this did not give very good practical results since the haemoglobin had a tendency to precipitate, thus rendering the solution unsuitable for satisfactory observations.

Alkaline haematin solutions are very similar to haemoglobin. There is a typical selective band centred at 4000 Å. which represents a slight shift towards the shorter wave-lengths. The general outline of the curve is unchanged. The small sharp peak at 3650 Å. also shifts towards the violet, and a broad band, which in centred at 3400 Å. in haemoglobin, appears at 3350 Å. The degree of absorption is about the same. The alkaline haematin in aqueous solutions gives a less clearly defined curve than the one in alcoholic solution. In aqueous solutions, the band centred at 4000 Å. spreads over the adjacent wave-lengths and includes the small curve normally present at 3650 Å. The other band is shifted considerably to the violet and is centred about 3200 Å. Since the haematin compounds are protein-free and have a spectrum similar to haemoglobin it follows that the globin fraction is not responsible for any of the observed bands in the haemoglobin spectrum with the exception of the one at 2700 Å. Acid haematin is similar to alkaline haematin, although in aqueous solution the band at 4000 Å. is broad and not well defined. Dissolved in alcohol its spectrum resembles closely the other haemoglobin spectra. Haemochromogens are very similar to the haemoglobin compounds, the band being centred about 4150 Å. This is in contrast with the haematin bands which are centred about 4000 Å. The physical state has an influence in the spectrum, apparently association of the molecules as in acid haematin causes shifting toward the shorter wave-lengths.

Haematoporphyrin, the iron-free pigment, has a spectrum which resembles in general form that of the other haemoglobin derivatives. This would indicate that the iron is not concerned in the spectrum. It has been postulated [Hicks & Holden, 1932] that the presence of the intense band at 4100 Å. is due to the fully saturated iron valence, but the intense band in the iron-free pigment seems to show such a hypothesis to be incorrect. The valence of the iron is apparently not a factor even in the compounds where iron exists in different states. This is shown by the comparison of the spectra of haemoglobin and methaemoglobin. There appears to be no essential difference in the general form of the curve and the intense band at 4100 Å. is present in both. Small differences in degree of absorption do not indicate much difference in chemical structure.

The bile pigment spectrum is different from that of haemoglobin. There is a region of heavy absorption from 5000 to 4000 Å. In alcohol and acetone there is a slight curve at 3400 Å., which does not appear in the ammoniacal solution. In the haemoglobin compounds and derivatives there must be a common feature which is responsible for the characteristic ultraviolet spectrum. In the bile pigment this structure or molecular arrangement is so changed that the spectrum is radically different. It has been shown that the haem radical is the common factor which apparently gives the characteristic spectrum which is not fundamentally altered by union with the globin, as in haemoglobin. Nor is it influenced by the valence of the iron as there is little difference between the spectra of oxyhaemoglobin and methaemoglobin and it is not altered radically by change in physical state, as in acid haematin in water solution. In the bile pigment, however, which is derived from haemoglobin by chemical rearrangement, the haem nucleus is no longer present. The porphyrin ring system made up of four substituted pyrrole nuclei is rearranged and altered to give four pyrrole nuclei, changed in substitution slightly and arranged in a straight chain. A study of the ultraviolet spectra leads to the conclusion that the absòrption band at 4100 Å. in haem compounds is due to the porphyrin ring arrangement and that the change of the ring to a straight chain involved in bile pigment formation from blood pigment is responsible for the lack of the specific absorption band at 4100 Å. in bile pigments.

SUMMARY.

1. Ultraviolet absorption curves have been described for oxyhaemoglobin, carboxyhaemoglobin, reduced haemoglobin, neutral and alkaline methaemoglobin, alkaline and acid haematin in aqueous and organic solvents, reduced alkaline haematin, pyridine and piperidine haemochromogens, haematopor-

phyrin, bilirubin in ammoniacal and organic solvents and also for native and denatured horse globin.

2. It has been shown that oxyhaemoglobin and its compounds and derivatives have a main ultraviolet band in the region of 4000 Å., a small band at 3750 Å. and a broad shallow band in the region of 3400 Å.

3. The band at 2700 Å. found in the haemoglobin compounds which still contain globin is due to the globin part of the molecule. Curves are presented for native and denatured globin solutions and there is only the band in the region of 2700 Å. There is very little spectral difference between the native and denatured globin.

4. Haematin compounds in organic solvents have greater ultraviolet absorption and the peak of the band is shifted towards the violet.

5. Bilirubin has a broad absorption band extending from 5000 Å. to about 4100 Å. There is no band at 3400 Å. The spectrum is quite dissimilar from that of the haemoglobin derivatives.

6. There is no basis for the theory that the intense band at 4000 Å. in haemoglobin compounds is related to the valence of the iron. Haematoporphyrin, an iron-free pigment, shows the band at 4000 Å.

7. It is believed that sufficient evidence is presented to show that the main violet absorption band in the region of 4000 Å. only occurs when the porphyrin pyrrole ring system is present and that it is absent when this ring system becomes a straight chain as in bilirubin.

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CCLXXXV. FAT METABOLISM IN FISHES.

X. HYDROGENATION IN THE FAT DEPOTS OF THE TUNNY.

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(*Received 24 September 1936.*)

Since the work of Leathes & Wedell [1909] and Hartley [1909] the idea of desaturation of fatty acids has been more or less accepted amongst biochemists, even although the original evidence for this theory has been considerably shaken. Strangely enough, the conception of the converse process, namely saturation or, to use a more usual term, hydrogenation, has until recent years been overlooked or ignored. This is the more surprising in that enzyme actions are considered as always reversible. Banks & Hilditch [1932] propounded the theory of hydrogenation of preformed glycerides in the fat depots of the pig, and this suggestion was strengthened and amplified in later papers from the same laboratory [Hilditch & Stainsby, 1935; Hilditch & Paul, 1935]. In the realm of aquatic fats the author adduced evidence suggesting hydrogenation and dehydrogenation processes in several cases [Lovern, 1932, 2; 1934]. As far as dehydrogenation processes are concerned numerous workers in recent years have established the presence of the necessary enzyme, although the reversibility of the system has not been demonstrated.

The author was fortunate, with the assistance of Dr F. S. Russell, of the Marine Biological Laboratory, Plymouth, in obtaining the whole of the viscera and a portion of the flesh of a large tunny (*Thynnus thynnus*) caught off Scarborough. As will be shown, the examination of this material afforded further evidence of hydrogenation as a mechanism controlling depot fat composition.

Experimental.

Fat was extracted from the flesh, liver, pyloric caeca, spleen and heart. In each case the fat was freed from phosphatides and the fatty acids of the glyceride fractions were examined in detail. The results are expressed in Table I, as

Table I. *Compositions of mixed fatty acids (weights %).*

	Saturated			Unsaturated				
Depot	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Flesh	4·2	18·6	3·5	—	6·2 (−2·7H)	26·0 (−3·2H)	23·5 (−5·5H)	18·0 (−6·8H)
Liver	Nil	17·9	8·9	—	3·4 (−2·5H)	23·5 (−2·8H)	28·2 (−5·5H)	18·1 (−7·4H)
Pyloric caeca	3·4	18·4	2·7	—	6·3 (−2·7H)	21·9 (−3·7H)	25·5 (−5·5H)	21·8 (−6·2H)
Spleen	Nil	21	7	—	7 (> −2·0H)	27 (−3·1H)	22 (−5·4H)	16 (−?H)
Heart	Nil	25	3	—	4 (> −2·0H)	26 (−3·4H)	25 (−5·4H)	17 (−7·5H)

weights %. From the heart and spleen the quantity of material available was only sufficient for examination by semimicro-methods, and in view of the probably greater experimental error involved the results for these two fats are only given to the nearest unit. The degrees of average unsaturation of the various groups are expressed as lack of hydrogen, as in previous papers.

In all the fats there was evidence of unsaturated acids of more than 22 carbon atoms. The quantities are probably of the order of 1–2 % but could not be determined with accuracy and are therefore included in the C_{22} percentages. In the liver and body fats traces of arachidic acid were found, but it appeared to be absent from the other specimens.

DISCUSSION.

These fats as a whole form a class with well marked peculiarities probably characteristic of the tunny family. Compared with the majority of marine species these are: for the saturated acids, high stearic and to a less extent high palmitic acid contents, and in three cases the absence of myristic acid: for the unsaturated acids, low palmitoleic acid content and entire lack of myristoleic acid (C_{14}) normally present in traces in aquatic fats.

Whilst these specific characters apply to the whole range of tunny fats, there are some marked variations within the series itself. The stearic acid content varies from 2·7 to 8·9 %. The degree of average unsaturation of the unsaturated C_{18} acids varies also, from $-2·8$ to $-3·7$ H. It will be seen from Table II that there is a steady and progressive relationship between these two phenomena. As the stearic acid content rises, so the degree of unsaturation of the remaining C_{18} acids falls. The figure for the heart fat, of course, is only reckoned to the nearest unit and probably only fortuitously fits so neatly, but the rest of the series is valid.

Table II. *Relation between stearic acid content and degree of unsaturation of C_{18} acids.*

Depot	Stearic %	Degree of unsaturation of C_{18} acids	Total saturated acid %
Pyloric caeca	2·7	−3·7	24·5
Heart	3	−3·4	28
Flesh	3·5	−3·2	26·3
Spleen	7	−3·1	28
Liver	8·9	−2·8	26·8

The C_{18} unsaturated acids consist of a mixture of oleic acid and polyethylenic acids. Thus a fall in the degree of average unsaturation means that the polyethylenic acids have been reduced in amount, or in degree of unsaturation. If a mixture of such acids and oleic acid is artificially hydrogenated with a nickel catalyst, the polyethylenic acids are preferentially saturated to oleic acid, before any stearic acid is produced. In hydrogenation processes in the animal, however, such is not the case, as was pointed out by Banks & Hilditch [1932] and confirmed in later work. There stearic acid may be produced from oleic while there is still much linoleic etc. acid present. The above series suggests that several reactions have been going on simultaneously—hydrogenation of oleic acid to stearic and hydrogenation also of the polyethylenic acids.

It seems almost certain that the food fat of the tunny would contain at most about 1 % of stearic acid. This is evident from what is now known as to the composition of a large range of aquatic fats and is further suggested by the composition of the pyloric caeca fat. It is in this fat that the stearic content is lowest. One of the main functions of the pyloric caeca, in the salmon at any rate, has been shown to be the absorption of food fat [Greene, 1913] and fat found in the caeca is likely to be largely food fat, probably already beginning to undergo modification to species requirements.

These requirements in the tunny appear to include the virtual elimination of C_{14} acids. In three of the fats there is no C_{14} acid at all. The effect of this is to diminish the available percentage of saturated acids. The tunny apparently spends considerable periods in relatively warm water and moreover is notable for having a body temperature some 3° higher than that of the water [Hanson, 1929]. Such a fish might perhaps be expected to have a higher total content of saturated acids than is usual in fishes, although it may be noted that such a condition was not found in the fat of some tropical carp examined by the writer [Lovern, 1935] as compared with British carp [Lovern, 1932, 1] inhabiting much colder water. Whilst the cause may thus be doubtful the fact remains that the tunny fats have higher contents of saturated acids than usual, as shown in the last column of Table II. The usual total content of saturated acids is from 15 to 20 %.

The evidence that this has been brought about by hydrogenation does not rest on the stearic acid figures alone. The palmitic acid percentage is higher than normal for fish fats (18 to 25 % as compared with a normal value of 8 to 16 %). That this is coupled with a lower palmitoleic acid content than normal is surely suggestive.

The total C_{16} acid percentage (21–29 %) is of the same order as that in most fish fats—i.e. in the tunny food fat—and it is evident that the high palmitic acid content has been at the expense of the palmitoleic acid content. Hydrogenation seems to be the only obvious explanation of such a condition. Finally the appearance of traces of arachidic acid in two of the fats is probably due to hydrogenation since this acid is not found in most aquatic fats. It is suggestive that the net effect of all these processes has been to keep the total saturated acid content roughly constant as is shown in the last column of Table II. The constancy is more marked if the fat from the pyloric caeca be excluded, and it has been mentioned that this fat is probably in process of being and is not yet completely adapted to the tunny requirements, further hydrogenation being required.

Constancy of total saturated acids in spite of varying proportions of the individual saturated acids has been encountered before [Lovern, 1932, 2] but is not found in all species [Lovern, 1934]. A possible case of simultaneous hydrogenation of oleic and polyethylenic C_{18} acids has been recorded before in ripening salmon eggs [Lovern, 1936] where, concurrently with a marked fall in the average unsaturation of the C_{18} acids, stearic acid appeared in increasing amount.

It may be noted that the greatest amount of hydrogenation appears to have gone on in the liver (highest stearic content, lowest degree of unsaturation of the C_{18} acids and presence also of arachidic acid). This is yet another instance from aquatic fats of a liver fat of composition opposed to that which the old idea of desaturation in the liver would lead one to predict. The conger eel affords an even better example [Lovern, 1934].

SUMMARY.

The fats from the flesh, liver, pyloric caeca, heart and spleen of the tunny (*Thynnus thynnus*) have been examined. The component fatty acids of all these fats exhibit peculiarities probably characteristic of the tunny family. They

include lack of C_{14} acids, low palmitoleic acid, high palmitic and stearic acid contents.

Apart from these common peculiarities the different fats show considerable variety in their contents of certain acids and in the degree of average unsaturation of the C_{18} acids. It is shown that the content of stearic acid is inversely proportional to the degree of unsaturation of the remaining C_{18} acids, and simultaneous hydrogenation of oleic and polyethylenic C_{18} acids is suggested as the explanation. It is further indicated from the results that hydrogenation of palmitoleic to palmitic acid takes place, as well as the production of traces of arachidic acid by hydrogenation of C_{20} unsaturated acids in two cases.

The purpose of the hydrogenation is possibly to maintain a roughly constant content of saturated acids. This is higher than usual for fish fats, perhaps because of the warm habitat of the tunny and the fact that its body temperature is somewhat higher than that of the water.

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CCLXXXVI. THE METABOLISM OF GALACTOSE.

III. 1. LACTOSE SYNTHESIS FROM (*a*) A GLUCOSE-GALACTOSE MIXTURE, (*b*) PHOSPHORIC ESTERS, BY SLICES OF THE ACTIVE MAMMARY GLAND *IN VITRO*.

2. THE EFFECT OF PROLACTIN ON LACTOSE SYNTHESIS BY THE MAMMARY GLAND.

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1 *a*. LACTOSE SYNTHESIS FROM A GLUCOSE-GALACTOSE MIXTURE BY SLICES OF THE ACTIVE MAMMARY GLAND *IN VITRO*.

SVANBERG [1930] investigated the possibility that a brei made of the lactating mammary gland might synthesize lactose from added glucose or a glucose-galactose mixture. In his experiments toluene was used as a preservative, and the reducing sugars were partitioned into disaccharide and monosaccharide fractions. With one doubtful exception, the results were negative. Michlin & Lewitow [1934] carried out similar experiments and claimed, on the basis of irregular changes in the total reduction of their solutions, to have demonstrated the synthesis of lactose from glucose and galactose mixtures. Grant [1935] determined the extent of lactose synthesis by slices of the active mammary gland immersed in oxygenated physiological salt solutions containing different hexoses. A quantitative micro-method was developed for the estimation of small amounts of lactose, using *S. fragilis* Jörg., as a biological reagent for this sugar. An increased synthesis of lactose was readily obtained with glucose as a substrate, while there was little evidence of such synthesis from fructose, mannose or galactose. In the present communication, the amounts of lactose formed *in vitro*, when glucose or galactose, respectively, is a substrate for slices of lactating tissue, are compared with those obtained from equivalent amounts of glucose-galactose mixtures.

EXPERIMENTAL.

2 ml. portions of an oxygenated physiological salt solution containing glucose (8 mg.), galactose (8 mg.), or a glucose-galactose mixture (4 mg. of each hexose), respectively, were incubated with approximately equal weights of mammary gland slices for 6 hours at 37°, as previously described [Grant, 1935]. At the end of this period the extent of the lactose synthesis was determined in each case; the reducing sugars were estimated by the differential fermentation method, as "fermentable sugar" (glucose, fructose or mannose), galactose and lactose.

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There was little evidence of increased utilization of galactose, when glucose-galactose mixtures were used, over the small amount found for galactose alone. The lactose synthesis was scarcely increased above the amount to be expected from the glucose component of the mixture (Table I).

Table I. *Lactose synthesis from glucose, galactose and glucose-galactose mixtures by the active mammary gland,* in vitro.

(6 hours, 37°.)

Wt. of tissue slices mg. (wet wt.)	Substrate	Hexose added mg.	Hexose remaining after 6 hrs. mg.	Lactose synthesized* mg.
172	Glucose	8	0·9	3·5
185	Glucose	8	0·4	3·0
160	Galactose	8	5·8	0·47
174	Glucose Galactose	4 4	0 2·8	2·1
103	Glucose Galactose	4 4	0·3 2·5	2·3

* Above that remaining after 6 hours in control experiments without added hexoses, which was 0·88–0·90 mg. lactose per 100 mg. tissue (wet weight).

These findings suggest that the mechanism of lactose synthesis in the lactating parenchyma does not simply involve a direct combination of free glucose and galactose in glucosidic linkage. In this connection, it was shown that a dried acetone-defatted powder of lactating mammary tissue did not contain an active β-galactosidase since it was unable to hydrolyse added lactose over a period of 120 hours (*p*H 7·3; 37°), but since the same preparation gave negative results for synthesis with glucose, or a glucose-galactose mixture the findings do not rule out the possible significance of this enzyme. Similar results suggesting the absence of an active lactase have been reported by Kleiner & Tauber [1932], using glycerol extracts of the lactating udder, and by Bradley [1912–13].

Experiments with non-living preparations of lactating mammary tissue.

Weinbach [1936] has stated that non-fermentable reducing material was synthesized from added glucose by a dried preparation of lactating glands of rats; in one of his two experiments, the amount of this material formed was in excess of the added glucose, and he believed that this excess came from a lactose-precursor which is non-reducing and only becomes available when the mammary gland preparations are placed in a solution containing glucose. The semi-quantitative method used for the identification of lactose and the absence of data concerning the amounts of lactose originally present in the preparations render an interpretation of the results very difficult.

In the present investigation, an attempt was made to obtain such active non-living preparations. Lactating glands of guinea pigs were rapidly frozen in CO_2 snow, powdered and suspended in physiological salt solution (*p*H 7·3) and kept in presence or absence of added glucose at 37° for periods of 0, 5 and 10 hours. Portions were removed at stated intervals and the glucose ("fermentable sugar"), galactose and lactose contents were determined in the deproteinized filtrates (Somogyi-$Zn(OH)_2$ precipitation) after suitable dilution by the method referred to previously. The results are shown in Table II. There was no synthesis of lactose in amounts which would convincingly demonstrate the presence of a non-reducing lactose-precursor in the gland preparations, or the ability of these

Table II. *Changes in sugar content of non-living preparations of lactating mammary tissue when kept at 37°.**

Exp. no.	Amount of mammary gland preparation g. in 10 ml.	Glucose (mg.) 0 hr.	Glucose (mg.) 5 hr.	Glucose (mg.) 10 hr.	Lactose (mg.) 0 hr.	Lactose (mg.) 5 hr.	Lactose (mg.) 10 hr.
Mammary tissue alone:							
1	2·2	0	1·6	0·6	11·8	10·8	11·5
2	2·5	0·4	2·8	0	5·8	9·2	7·5
3	3·8	0	4·2	4·0	30·2	36·5	35·0
Mammary tissue + added glucose:							
1	2·2	194	192	195	12·8	13·8	13·5
2	2·5	188	199	159	6·8	0	2·5
3	3·8	189	192	184	37·5	38·5	46·3

* Galactose was not present in measurable amounts in either series. Exps. 1, 2 and 3 represent preparations of mammary glands from three different animals; the glands were removed from one to two hours after the last suckling period.

preparations to convert added glucose into lactose. Small amounts of "fermentable sugar" (glucose, fructose or mannose), were produced, but galactose was not present in recognizable amounts. A preparation from mammary tissue by the method used by Meyerhof in preparing active glycolytic extracts from muscle also gave negative results for lactose synthesis from added glucose.

It has been suggested by Helferich [1933] that the process by which a monosaccharide is converted into a disaccharide in the presence of a synthetic catalyst must be regarded as precisely similar to that by which α- and β-glucoses are converted into the two methylglucosides. Thus according to classical theory the synthesis would occur in two steps:

$$(1)\quad \alpha\text{-glucose} \rightleftharpoons \beta\text{-galactose};$$

$$(2)\quad \alpha\text{-glucose} + \beta\text{-galactose} \xrightleftharpoons{\beta\text{-galactosidase}} \underset{\substack{(4\ (\beta\text{-galactosido-})\\ \alpha\text{-}d\text{-glucose})}}{\text{lactose}} + H_2O.$$

The results obtained in the present investigation are not those one would expect if a rapid direct coupling of glucose and galactose in glucosidic-linkage occurs as part of the mechanism of the lactose synthesis in the mammary gland (reaction 2).

1*b*. PHOSPHORIC ESTERS AS SUBSTRATES FOR LACTOSE SYNTHESIS BY SLICES OF THE ACTIVE MAMMARY GLAND *IN VITRO*.

The important role of phosphoric esters in the intermediary carbohydrate metabolism of yeast and muscle has been well established by the extensive researches of Harden, Robison, Meyerhof, von Euler, and their co-workers [see Robison, 1936]. It is possible that these esters may also play a part in the changes taking place during the conversion of glucose into lactose in the lactating mammary gland. It is known that preparations of the active gland produce lactic acid from glucose, galactose, lactose and phosphoric esters and that here, as in yeast and muscle, the phosphorylation taking place is very likely connected with the active glycolysis of the lactating parenchyma [Svanberg, 1930; Barrenscheen & Alders, 1932; Börst, 1932; Brenner, 1932; Folley & Kay, 1935;

Grant, 1935]. No evidence, however, has yet been presented which directly connects the phosphorylation with the synthesis of lactose.

In an attempt to obtain further information on this point, some of the esters which are known to play an important part in the fermentative and glycolytic processes, and which have been shown to be produced during the fermentation of galactose by preparations of adapted yeast [Grant, 1935], have been used as substrates for the synthesis of lactose by slices of the active mammary gland *in vitro*. The following naturally occurring hexosephosphates were employed: glucose-6-phosphate, fructose-6-phosphate, fructose-1:6-diphosphate; the synthetic galactose-6-phosphate which has not been found among the products of galactose fermentation has also been used. In view of the importance of the 3-carbon ester, phosphoglyceric acid, in the later stages of the glycolytic process [Lohmann & Meyerhof, 1934; Meyerhof & Kiessling, 1935], this ester has also been included as a substrate.

EXPERIMENTAL.

The general conditions are those described in Section 1 *a*, suitably altered to enable the sugar analyses, "fermentable sugar" (glucose, fructose and mannose), galactose and lactose, to be made by the differential fermentation method on the ester-free deproteinized tissue filtrates. The neutral sodium salts of the esters used as substrates for the mammary tissue slices were added in equimolar amounts to the glucose used in the comparative experiments, except for the phosphoglyceric acid, for which twice the molar equivalent was employed. The esters remaining at the end of the experimental period were precipitated at *p*H 8·2 by the addition of barium acetate solution to the alcohol-deproteinized tissue filtrates.

Table III. *Lactose synthesis by slices of the active mammary gland* in vitro *at 37°. Availability of phosphoric esters as substrates.*

Time hours	Substrate	mMol. (× 10³)	mg. lactose per 100 mg. tissue (dry wt.)			% substrate decomposed in 6 hours†		
			I*	II			II	
0	Control	—	2·0	1·1			—	
6	Control	—	1·4	3·5			—	
6	Control	—	2·1	2·2			—	
6	Phosphoglycerate	88	2·8	2·4			30	
6	Phosphoglycerate	88	2·3	2·7			43	
6	Phosphoglycerate	88	3·7	3·1			40	
6	Glucose	44	6·5	10·8			92	
6	Glucose	44	7·2	14·7			94	
			III	IV	V	III	IV	V
0	Control	—	—	1·8	—	—	—	—
6	Control	—	3·3	2·7	—	—	—	—
6	Control	—	3·9	2·0	1·9	—	—	—
6	Glucose-6-phosphate	44	1·2	2·1	1·6	54	24	52
6	Galactose-6-phosphate	44	2·4	—	1·0	60	—	63
6	Fructose-6-phosphate	44	1·8	2·1 4·5	1·0	53	40 24	20
6	Fructose-1:6-diphosphate	44	1·5	—	0·8	38	—	15
6	Glucose	44	6·6	11·5	—	83	77	—
6	Glucose	44	—	21·6	5·8	—	85	100

* I, II, III, IV and V represent separate experiments with lactating glands from different animals.

† When the substrate is a phosphoric ester, the value is calculated as the % ester-P set free as inorganic phosphate; when the substrate is glucose the value is calculated as the % decrease in "fermentable sugar".

Table IV. *Action of mammary tissue slices on hexosephosphates.*

Esters originally present in equivalent amounts to the glucose (44×10^{-3} mMol.) i.e. 8 mg. hexose. Exps. III and V are those referred to in Table III.

	Hexoses present after 6 hours at 37°			
	"Fermentable sugar"*		Galactose	
Substrate	Exp. III	Exp. V	Exp. III	Exp. V
Glucose-6-phosphate	0·6	0·3	0	0
Galactose-6-phosphate	0	0	2·7	1·6
Fructose-6-phosphate	1·1	0·3	0	0
Fructose-1:6-diphosphate	1·0	0·8	0	0

* Glucose, fructose or mannose.

Aliquot portions of the ester-free alcoholic solutions were evaporated to dryness at 60° and the aqueous solutions of the residues analysed for "fermentable sugar" (glucose, fructose or mannose), galactose and lactose. The results obtained are summarized in Tables III and IV.

The phosphoric esters were decomposed to varying degrees in the different experiments (15–63 %), as judged by the inorganic phosphate liberated. There was little evidence of an increase in lactose with the esters as substrates, in comparison with that occurring when glucose was employed. Nor was there any accumulation of free galactose, except in the experiments with galactose-6-phosphate, where it arose, presumably, by the hydrolysis of this ester. The varying amounts of free glucose in the glucose-6-phosphate experiments and of free fructose in those with the fructose esters very likely had a similar origin (Table IV). There was no evidence of the synthesis of free hexoses from the phosphoglyceric acid. These negative results are, however, not conclusive since there is always the possibility that the esters were unable to penetrate into the tissue cells.

Effects of fluoride and iodoacetate on lactose synthesis.

Sodium fluoride (0·04 *M*) and sodium iodoacetate (0·02 *M*) almost completely inhibited the utilization of added glucose by the mammary gland slices. The glucose was recovered unchanged to the extent of 93–96 %, in the experiments with sodium fluoride, and 88–92 % in those with sodium iodoacetate; lactose synthesis was stopped, and there was no evidence of the production of free galactose. It is not clear whether the lactose mechanism is inhibited independently, or whether this inhibition is consequent upon the effects of these two compounds upon the glycolytic process.

2. THE EFFECT OF PROLACTIN ON LACTOSE SYNTHESIS BY THE MAMMARY GLAND.

Nelson [1936] has reviewed the numerous researches of the last decade relating to the hormonal control of the mammary gland. It has been possible to correlate the proliferative changes of duct and lobule-alveolar development observed during the growth phase with the action of oestrin, possibly supplemented with progesterone. It is suggested that the accumulation of the products of secretion, and the production of milk, characteristic of the secretory phase, are due to the lactogenic hormone of the anterior lobe of the pituitary. Contradictory evidence has been reported as to whether the oestrogenic hormones can cause duct and lobule-alveolar development in hyphophysectomized animals [Nelson, 1936; Reece *et al.*, 1936]; Gomez & Turner [1936, 1] state that in complete absence of the hypophysis, no growth of the mammary parenchyma is observed. Oestrogenic substances can cause responses in the same mammary gland varying from duct growth and alveolar dilatation to a hyperplasia resulting in traumatic oedema and mammary carcinoma [Collip *et al.*, 1936; Gardner *et al.*, 1935; Burrows, 1935; Pallot, 1936; Lacassagne, 1936]. In contrast to the earlier results obtained with crude extracts of the anterior pituitary, evidence is accumulating that the purified "lactogenic" hormone preparations, while very active in causing proliferation of the crop-gland of the pigeon, do not serve in their lactogenic effect as a complete replacement for the secretion of the animal's own pituitary [Gomez & Turner, 1936; Nelson & Gaunt, 1936, 2].

Evans [1936] found that an alkaline extract of the anterior pituitary caused up to 50 % increase in milk production of lactating cows and goats but had no effect in bringing back into lactation animals which had been allowed to "go dry" for this purpose. De Fremery [1936] obtained only a few drops of milk secretion in virgin goats even though large amounts of prolactin were used; he suggested that prolactin only causes milk secretion in normal quantities after a preliminary action of oestrogenic hormones [see also, Gomez & Turner, 1934; Lyons & Catchpole, 1933].

While prolactin undoubtedly causes the production of variable amounts of a fluid which has the physical appearance of milk, very few investigations so far reported have been concerned with the nature of this induced secretion. For this reason, an investigation was made of the effect of the "lactogenic" hormone on the synthesis of lactose—since this is a specific mechanism of the active mammary gland.

EXPERIMENTAL.

Normal multiparous guinea-pigs were separated from their litters (an equal number of 3 young to each parent) two weeks after the onset of lactation. The involution of the mammary glands was allowed to proceed for several days (stripping the glands twice daily), until only small amounts of lactose-free serous fluid were being produced. At this stage prolactin in aqueous solution was injected subcutaneously, in amounts varying from 2·5 to 16·6 mg. per 100 g. body weight, per day. After a variable period (1 to 3 days), during which the regressing glands secreted small amounts of a cloudy fluid, the secretion of "milk" was reinstated. The milk was collected twice daily in a graduated capillary pipette and washed into 50 % alcohol. After centrifuging, the alcoholic centrifugate and washings were evaporated to dryness on a water-bath at 60°, and the residue was taken up in water to a suitable volume. The lactose content of this aqueous solution was determined by the differential fermentation method [Grant, 1935]. The total protein figures represent the dry weight of the washed precipitate. In some cases, the milk was deproteinized by the Somogyi-$Zn(OH)_2$ precipitation method, both methods of deproteinization yielding the same lactose value.

Examined under identical conditions in a capillary pipette the "milk" had a yellowish white colour compared with the chalky white colour of the normal milk of the guinea-pig. The microscopic examination revealed numerous fat globules and colostrum corpuscles. The results of the chemical examination are shown in Table V. Lactose was the only sugar present in the "milk" and in abnormally low concentration (0·04–0·24 %). The normal milk of the guinea-pig in early

Table V. *Lactose synthesis,* in vivo, *reinstated by prolactin in the mammary glands of normal multiparous guinea-pigs, which have just ceased lactation.*

Animal no.	Prolactin injected mg./100 g. per day	No. days	Total milk secretion ml.	Lactose synthesized		Total protein	
				mg.	%	mg.	%
1	3·0*	14	0·24	0·5	0·2	19·6	8
2	3·3†	5	0·11	0·2	0·2	6	5·5
3	3·3†	5	0·07	0·1	0·14	8	11
4	3·3*	6	0·28	0·1	0·04	—	—
	2·5*	3	0·20	0·2	0·1	—	—
	0	9	0·17	0·4	0·24	21	12
5	16·6‡	5	0·16	0·2	0·1	—	—
Normal animals:							
6	0; late lactation 3 hours		0·30	4·6	1·5	15	5
7	0; 1st day lactation 3 hours		0·9–	40·2	4·5	—	—
	0; 2nd day lactation 3 hours		0·9–	49·8	5·5	—	—

Prolactin preparations:

* Prolactin, Allen and Hanbury, Ltd.; 1 g. obtained from approximately 644 g. of fresh anterior pituitary.

† The fraction insoluble in 86 % alcohol at pH 6, prepared from frozen fresh anterior pituitaries by the method of Bates & Riddle [1935].

‡ The fraction insoluble at pH 6·4, prepared from acetone-dried anterior pituitary powder by the method of Lyons & Catchpole [1933].

lactation contains 4·5–5·5 %, and that of late lactation 1·5 % of lactose. The maximum induced milk secretion was 0·08 ml. per day, compared with 0·3 ml. per 3 hours in the final stages of lactation, and over 0·9 ml. per 3 hours on the first and second days of lactation. Though the stimulated mammary glands of the animals were tender, the animals did not undergo significant weight changes during the injection periods.

Individual mammary glands varied greatly in the amounts of milk produced and in the time taken for this to replace the secretion of cloudy serous fluid. A preparation of prolactin (Allen and Hanbury) assaying 1·8 crop-gland units per mg., gave similar results to those recorded in Table V. In doses of 12 mg. per 100 g. body weight per day, injected subcutaneously into female guinea-pigs in the last stage of lactation, this preparation increased the milk secretion from 0·02 to 0·15 ml. per day. Injected for two weeks into animals which had gone completely "dry" it induced only cloudy serous fluid, and in both the secretion and the mammary glands of these animals the sugar was not lactose but glucose ("fermentable sugar"). The preparations of prolactin used were inactive in causing a measurable increase in lactose synthesis from added glucose *in vitro,* but this is not surprising considering the limited effect of these preparations *in vivo.*

DISCUSSION.

Evans [1933], using an alkaline extract of the anterior pituitary, obtained an induced secretion resembling colostrum in its appearance from a virgin goat, but no mention is made of its lactose content. Catchpole *et al.* [1933] reported that the milk secretion induced by prolactin in a virgin heifer had a low lactose (total reduction) and high chloride content. De Jongh & Dingemanse [1931] stated that the milk obtained from the mammary glands of normal male guinea-pigs, after the cessation of the injection of large amounts of oestrin, possessed a sugar content (total reduction) of 17–21 %, and an "albumin" content of 17–24 % of the total dry residue.

In the present experiments, *in vivo* synthesis of lactose was re-established in the inactive regressing glands of the female guinea-pigs by the stimulus of prolactin, though to a very limited extent. The secretion obtained had the physical appearance of milk but an abnormally low lactose content. The mammary parenchyma of the glands at the time of injection of the prolactin was probably in the intermediate stage of involution, consisting of secreting, regressing and regressed acinar tissue [see de Fano, 1922; Loeb & Hesselberg, 1917]. Apparently these regressing glands must be subjected to additional influences to that afforded by prolactin, before they can again resume either normal milk production or the synthesis of lactose in normal quantities. This is probably not due to a lack of proliferated alveolar tissue but to the need of most of the acinae for additional hormonal stimuli, very likely those of oestrone and progesterone. The results obtained by de Fremery, mentioned above, suggest that such may be the case, and recently Allen & Heckel [1936] found that oestrin prevents the regression of the mammary glands which normally takes place in the pseudopregnant animal and also causes a thickening of the glands (when oestrin injections are continued after castration) to a stage characteristic of the preparturient normal female.

The results obtained in the present investigation offer additional evidence in support of the view that the actual process of milk secretion (and of the concomitant lactose synthesis) depends upon the simultaneous action of a number of different hormones, rather than being conditioned solely by the action of prolactin. The methods at present in use for the assay of prolactin depend on glandular proliferation and upon the quantity and physical appearance of the milk and neglect the composition of this induced secretion. Nelson [1936] has called attention to the fact that variations between the pigeon "crop-gland" unit and the mammary gland "lactogenic" unit for rabbits, may be between 15–1 to 100–1, with an average of 40–1. The present findings indicate the importance of determining the chemical composition of the induced secretion in the investigation or assay of the lactogenic property of hormones.

SUMMARY.

1. When glucose-galactose mixtures are employed as substrates for lactating mammary tissue *in vitro* the lactose synthesis is not noticeably increased beyond that for glucose alone. This result is inconsistent with the view that a rapid direct coupling of galactose to glucose in glucosidic-linkage represents the mechanism employed by the active mammary gland in the synthesis of lactose. There was no evidence of an active β-galactosidase in preparations of the lactating gland. Attempts to obtain non-living preparations able to synthesize lactose from glucose, or from a glucose-galactose mixture, were unsuccessful.

2. The following phosphoric esters, glucose-6-phosphate, fructose-6-phosphate, fructose-1:6-diphosphate, galactose-6-phosphate and phosphoglyceric acid did not yield increased synthesis of lactose with slices of lactating mammary gland *in vitro* although the slices were active when glucose was the substrate. Sodium fluoride (0·04 M) and sodium iodoacetate (0·02 M) inhibit almost completely both glucose utilization and lactose synthesis by slices of lactating tissue.

3. The *in vivo* synthesis of lactose was re-established to a limited extent in the inactive regressing mammary glands of female guinea-pigs by the stimulus

of prolactin. The induced secretion, slight in amount, resembled milk in its physical appearance but possessed an abnormally low lactose content (0·04–0·24 %). The presence and concentration of lactose, a specific product of the lactating mammary parenchyma, indicated to what extent the "lactogenic" stimulus was a physiological one. Prolactin, alone, is unable to reinstate normal milk production in the regressing mammary gland of the female guinea-pig.

I wish to take this opportunity to thank Prof. R. Robison for his interest and advice during this investigation, and to the Lister Institute for providing laboratory facilities. My thanks are also due to Dr Norman Evers, of Messrs Allen and Hanbury, Ltd., for generous supplies of prolactin preparations and to Dr M. Laskowski for the gift of a kilogram of desiccated anterior pituitary powder.

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CCLXXXVII. THE IMPORTANCE OF "PANTOTHENIC ACID" IN FERMENTATION, RESPIRATION AND GLYCOGEN STORAGE.

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WHILE the primary research dealing with the isolation and chemical characterization of "pantothenic acid" [Williams *et al.* 1933; Williams & Saunders, 1934] has engaged our study for some time, we have also carried out certain experiments which give a clue as to why this substance is universally present in tissues whether of plant, animal or bacterial origin.

If the yeast with which we have been dealing [Williams *et al.* 1933] is seeded sufficiently heavily into a synthetic medium or better into one containing a very little added pantothenic acid, a crop of yeast large enough for experimental use may be obtained. Repeated experiments in which such yeast was grown and extracted have shown that pantothenic acid is not produced as the yeast grows (slowly), but on the contrary the yeast crop and medium after growth together seem to contain somewhat less pantothenic acid than was present before growth took place. Yeast grown under such conditions may have a pantothenic acid content as low as 2 % of that present in "normal" yeast.

This yeast is almost lacking in fermenting power as compared with yeast grown under favourable nutrient conditions. It is capable of bringing about a much increased fermentation, however, if a minute amount of a pantothenic acid preparation is added directly to the sugar solution in which the yeast is suspended. Under these conditions yeast multiplication does not take place as determined by our thermocouple method [Williams *et al.* 1929].

The results of a 21-hour fermentation are indicated in Table I. With cruder pantothenic acid preparations similar results were obtained. The largest amount of pantothenic acid added in this particular experiment was evidently an overdose. It is many times that which would be present in 80 mg. of yeast grown under ordinary conditions.

Table I. *Effect of pantothenic acid on fermentation.*

Weight of yeast (mg.)	Sucrose solution (ml.)	Pantothenic acid preparations added (γ)	Volume CO_2 produced (ml.)
80	25	0	0·1
80	25	1 γ (2 units)	0·45
80	25	10	1·60
80	25	100	0·55

An even more striking increase in the fermenting power of this deficient yeast was obtained when a cold water extract of fresh yeast (Fleischmann's) plus a pantothenic acid preparation were together added to the fermentation mixture. The effect of this addition, which was also noted when glucose was the substrate used, is not always uniform. It is well known from the researches of Euler and others that at least one agent other than the classical enzyme and coenzyme is involved in the fermentation process. Our experiments clear up this complex situation only to the extent of demonstrating that pantothenic acid is an indispensable factor.

The aerobic respiration of the deficient yeast is also very low as compared with that of normal yeast. The rate is increased only very little when pantothenic acid is added directly to the yeast after it is grown, but yeast grown with a good supply of pantothenic acid respires much more rapidly than the deficient yeast, which is grown under otherwise identical conditions. These experiments seem to point unmistakably to the conclusion that pantothenic acid is necessary for carbohydrate utilization whether aerobic or anaerobic.

We call attention to the fact also that in alfalfa seedlings stimulated in growth by pantothenic acid [McBurney *et al.* 1935] the amount of carbohydrate was about doubled whereas the nitrogenous constituents were not increased at all, again indicating a relationship of pantothenic acid to carbohydrate metabolism.

Finally we cite the recent experiments of McAnally & Smedley-MacLean [1935] in which it was shown that glycogen storage in yeast is greatly aided by maltose. From our previous experience with maltose and from experiments carried out since the above paper was published it is clear to us that maltose as such is probably not the active agent which promotes glycogen storage but that pantothenic acid is at least one of the factors involved. That maltose (as purchased) is always effective, and sometimes exceedingly so, in stimulating yeast growth is shown by the following series of results when the 2 % sucrose in our ordinary medium [Williams & Saunders, 1934] is replaced by different brands of maltose.

Table II. *Different samples of maltose as yeast stimulants.*

Sugar used	Yeast crop (duplicates) mg./ml.
Sucrose, Baker's C.P.	0·020 0·019
Maltose, tech. Difco	0·040 0·039
Maltose, tech. Pfanstiehl	0·55 0·57
Maltose, Difco, recrystallized and dialysed	0·0375 0·038
Maltose, Difco, twice recrystallized and dialysed	0·025 0·027
Maltose, Difco, "standardized"	0·0325 0·0330
Maltose, electrically purified (see below)	0·015 0·014

In another experiment Eastman's maltose was shown to be about on a par with the "standardized" Difco brand. The electrically purified sample was purified exhaustively by fractional electrical transport [Williams, 1935] and is a product such as is not obtainable otherwise. Its response shows that maltose itself is somewhat less effective as a carbohydrate source than high grade sucrose.

To show that the electrolytic treatment actually removed pantothenic acid from the maltose (Eastman brand 5·0 g.) the results in Table III are cited. It is

Table III. *Electrolytic purification of maltose.*

Sugar used	Yeast crops (mg./ml.)
Maltose, Eastman	0·040 0·039
Same purified electrolytically	0·0125 0·0125
Cell I, electrolytic system pH 3 1/8 of cell contents used	0·375 0·375
Cell II, electrolytic system pH 4 1/8 of cell contents used	0·475 0·475

characteristic of pantothenic acid to migrate to the position where it migrated in this experiment.

We have tried four different samples of maltose and have found that all of them contain pantothenic acid in very appreciable amounts. From a consideration of the sources and the methods of preparation and purification of maltose it is not surprising that this is true. The fact that maltose contains pantothenic acid and that its removal (by electrolytic transport) leaves maltose which is not effective in yeast growth stimulation led us, with other facts, to suspect that maltose itself does not promote glycogen storage as observed by McAnally & Smedley-MacLean but that pantothenic acid is a factor in bringing about this result. In order to investigate this possibility the experiments summarized below were carried out using the methods of the above authors.

These investigators do not give definite information as to the ratio of the weight of yeast used to the volume of the solution in which it was incubated. They do, however, indicate that 2 g. of yeast were incubated in 125 ml. in a fermentation experiment. In order to obtain results which might be on the same basis as theirs we incubated 200 mg. of yeast in 12·5 ml. of solution. The smaller amount was used because of the greater ease involved in making the determinations and the smaller supply of maltose required. The electrolytic purification of large amounts of maltose is time-consuming.

The yeast used was a Munich bottom yeast obtained from the Salem (Oregon) Brewery Association. The yeast was freed from the last trace of wort by suspending in a large volume of water and centrifuging; this procedure was repeated eight times. The sugar solutions were made up of such concentration that, after the addition of the phosphate and pantothenic acid solutions, a 5 % concentration of sugar was obtained. The dosage of pantothenic acid was 4γ per sample indicated. This is about twice the amount contained in 0·6 g. of Eastman's maltose. The phosphate concentration was 0·1 %, the same as used by McAnally & Smedley-MacLean. Four incubation tests were run with each sugar; two were used to determine the total carbohydrate of the yeast and two for the glycogen, using the method of Mayer. Bertrand's method was used in all sugar determinations.

All values in Table IV are in g. of sugar as glucose per 10 g. of original moist yeast. The descriptions given of the media used are self-explanatory. The abbreviation P.A. stands for pantothenic acid. The yeast was incubated for 48 hours at 30°.

From the evidence presented it is clear that pantothenic acid is an important factor in the storage of glycogen as well as being effective in increasing the total carbohydrate content of the yeast.

We realize, however, that different strains of yeast, though similar in nature, often present marked differences in behaviour. In order to make our conclusions

Table IV.

No.	Medium	Total carbohydrate (g. glucose per 10 g. original yeast)	Glycogen (g. glucose per 10 g. original yeast)
0	Water (distilled)	0·45	0·214
0	,,	0·45	0·214
1	Glucose (Merck)	0·945	0·228
2	,,	0·945	0·292
3	Glucose + PO_4	1·07	0·349
4	,,	1·09	0·362
5	Glucose + P.A.	1·06	0·362
6	,,	1·045	0·332
7	Glucose + PO_4 + P.A.	1·15	0·382
8	,,	1·11	0·363
9	Maltose (electrolytically purified)	0·935	0·271
10	,, ,,	0·910	0·258
11	Maltose + PO_4	1·06	0·338
12	,,	1·07	0·347
13	Maltose + P.A.	1·02	0·337
14	,,	1·045	0·331
15	Maltose + PO_4 + P.A.	1·08	0·395
16	,,	1·08	0·382
17	Maltose (Eastman's)	1·24	0·462
18	,,	1·26	0·462
19	Maltose + PO_4	1·35	0·472
20	,,	1·38	0·485
21	Maltose + P.A.	1·36	0·462
22	,,	1·33	0·445
23	Maltose + PO_4 + P.A.	1·40	0·53
24	,,	1·44	0·505
25	Maltose (Pfanstiehl's) tech. crys.	1·62	0·585
26	,, ,,	1·66	0·575
	Original yeast	0·86	0·265

more general we have performed similar experiments using Fleischmann's "XR" yeast instead of the brewer's yeast. The results were of the same order and led to identical conclusions. The differences in the values between the two yeasts were accounted for by the different initial glycogen contents.

These results are in line with other direct evidence which we have cited indicating that pantothenic acid plays some fundamental role in carbohydrate anabolism and catabolism, whether the latter is aerobic or anaerobic in nature. It is interesting in this instance to recall that the pantothenic acid concentration in mammalian tissue is the greatest in the liver and red striated muscle [Rohrman *et al.* 1934]. This is a suggestive fact as these tissues are pre-eminently involved in glycogen deposition.

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CCLXXXVIII. THE ROLE OF THE 4-CARBON DICARBOXYLIC ACIDS IN MUSCLE RESPIRATION.

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THE oxidation of dicarboxylic acids in muscle was studied by Battelli & Stern [1911; 1914], Thunberg [1918; 1923] and other workers, whilst Einbeck [1913; 1914] demonstrated the presence of succinic and fumaric acids in perfectly fresh muscle tissue.

The dynamic part played by these acids in muscle respiration was first studied by Mrs Needham [Moyle, 1924; Needham, 1927] who followed the changing concentrations of succinic, fumaric and malic acids in muscle under various conditions, finding that the total concentration of these acids rises in anaerobiosis and falls on oxygenation, and that the succinic acid maximum is renewed under anaerobic conditions from some source other than fumaric and malic acids.

An increased oxygen uptake on addition of fumaric acid to muscle after various degrees of washing was demonstrated by several workers [Thunberg, 1909, 1911; Meyerhof, 1919; Grönvall, 1924] and Gözsy & Szent-Györgyi [1934] also observed that the increased oxygen uptake after addition of fumaric acid to minced muscle tissue was inhibited by malonic acid. From these and other observations Szent-Györgyi *et al.* [1935] formulated a theory which, in its final published form, assigns to fumaric acid the role of an essential catalytic link in the chain of reactions which composes the chief respiratory system of muscle. Szent Györgyi *et al.* suggest that, in this system, the fumaric acid is oxidized by the Warburg-Keilin system to oxaloacetic acid and that this is reduced by the substrate dehydrogenase systems plus substrates to succinic acid which becomes reoxidized to fumaric acid.

In an attempt to confirm this theory the present experiments were divided into two sections.

(*a*) *With unwashed tissue.* Respiration experiments were carried out, a known quantity of fumaric acid being added to the suspension fluid of the tissue in some cases, but not to the controls. Accurate methods of estimation of small quantities of fumaric acid and its possible oxidation and hydration products were found and an attempt was made to draw up a balance sheet for each experiment showing the oxygen used in respiration, the fumaric acid remaining and the succinic, oxaloacetic, pyruvic and malic acids produced. Thus it could be seen whether any actual disappearance of fumaric acid occurred, or whether the acid was present at some stage of the fumarate-oxaloacetate-succinate-fumarate cycle, or as the hydration product malic acid.

(*b*) *With washed tissue.* Experiments were done in which cozymase, fumaric acid and a substrate were added to the washed tissue. Estimations were carried out as before, and a balance sheet was drawn up to see whether the fumaric acid was catalysing the oxidation of the added substrate or merely itself being consumed as a substrate for oxidation.

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EXPERIMENTAL METHODS.

The oxygen uptake was measured in Barcroft manometers, the CO_2 evolved being absorbed by frilled filter-papers soaked in 5% KOH. The suspension fluid was *M* phosphate buffer at *p*H 7, and the solutions of the acids added (neutralized with NaOH) were 0·1 *M*. The volumes of these solutions and water put into the vessels of the manometers were such that the final concentration of the fluid was 0·099 *M*, which is approximately isotonic with the muscle; this is important as Greville [1936] shows that the respiration of minced muscle varies with the tonicity of the suspension fluid.

The manometers were shaken in a water-bath, the temperature of which was thermostatically controlled at 37·5°.

Preparation of the muscle.

(*a*) *Unwashed tissue.* The pigeon breast muscle used in these experiments was prepared according to the directions given by Szent-Györgyi *et al.* [1935], everything being done as rapidly as possible after death and all the implements, vessels and solutions used being ice-cold. Three methods of weighing out the minced tissue were tried, and the following proved most satisfactory, allowing for the greatest speed in preparation of the tissue: 4 g. of the minced muscle were weighed out and transferred to an ice-cooled mortar in which they were mixed carefully with 26 ml. of buffer. The suspension was rapidly stirred and 3 ml. (containing 0·4 g. of muscle) were pipetted into each manometer vessel.

(*b*) *Washed tissue.* The breast muscle was prepared as before and the 4 g. of muscle were transferred to a flask and shaken vigorously with twenty times the volume of ice-cold distilled water. After 2 min. shaking the suspension was filtered through muslin and squeezed well. The muscle was returned to the flask and shaken with a fresh volume of ice-cold water, being allowed to stand for 5 min. in ice before filtration and squeezing. The washed tissue so obtained was transferred to a mortar, suspended in buffer and pipetted out into the manometer vessels in 3 ml. amounts as above.

Solutions to be added to the respiring tissue after equilibration of the manometers in the water-bath were put either into a side-tube of the manometer vessel or into a Keilin cup suspended in the vessel. The final volume of the suspension plus all additions was 4 ml. and a similar volume of buffer was put into the other cup of the manometer.

In early experiments the vessels were filled with air, but in more recent ones the apparatus was evacuated and filled with oxygen. Duplicate experiments were run simultaneously in every case, each duplicate consisting of three manometers with fumarate added to the respiring muscle and one without addition which acted as a control. Measurement of the rate of respiration was started 20 min. after death with unwashed tissue. The experiments were run for times varying from 1 to 2 hours.

At the end of the respiration experiments the vessels were detached from the manometers and the muscle was immediately precipitated by the addition of 0·8 ml. of 20% trichloroacetic acid (rendering the total concentration of trichloroacetic acid 4%). The filter-paper was removed from the central cup of the vessel and any remaining potash was blotted up with strips of dry filter-paper. The contents of the three vessels of each duplicate experiment, containing additions of fumarate or fumarate plus other substrate, were filtered together through a small filter-paper into a glass evaporating dish, whilst the contents of the vessels containing the control muscle from each set of duplicates were also filtered together. The vessels were carefully rinsed with successive small quantities of 2 % trichloroacetic acid which were then used to wash the muscle on the paper. The muscle was also ground up in a mortar with 2 % trichloroacetic acid, being refiltered on the same paper. The final volume of the fluid in the evaporating dish after intensive washing of the vessels, mortar, muscle and paper, was about 30 ml. This was then used for the estimations described below.

The amounts of dicarboxylic acids and others to be estimated were generally between the limits 0·1–6 mg., as 2 mg. of fumaric acid were added to 400 mg. of tissue. Experiments were done to test each method, using the concentrations of the particular acid ranging between the limits, adding it to 0·4 g. of the minced muscle in phosphate buffer and proceeding as in the experiments above after measurements of the respiration. Results showed that the methods used were accurate to a degree which allowed the amounts of substances estimated after respiration experiments to be regarded as significant.

Estimation of the dicarboxylic acids.

The method used was a modification of that described by Needham [1927].

(*a*) *Fumaric and succinic acids.* The trichloroacetic acid extract from the muscle was evaporated on a boiling water-bath until the volume was reduced to less than 10 ml. This was transferred to a separating funnel and the evaporating dish was rinsed out with a few ml. of distilled water which were added to the bulk of the fluid. Four successive extractions with dry ether were made, the volumes used being 20, 15, 20 and 15 ml. The ether and fluid were shaken vigorously for 2 min. during each extraction and, after separation, the ether was filtered through a dry pleated filter-paper into a flask containing 5 ml. of distilled water, and the ether was distilled off. A drop of phenol red was added to the remaining solution and *N* NaOH was run in to bring its *p*H to 8 (deep pink to phenol red). 0·2 ml. of 25 % barium acetate solution was added to precipitate any phosphate which had been extracted by the ether, the solution was transferred to a centrifuge-tube and the precipitate was spun down. The centrifugate was filtered through a small paper into a glass dish containing a few drops of dilute nitric acid to bring the *p*H of the filtrate just to the acid side (otherwise a film of barium carbonate formed on the surface and interfered with the silver precipitation). The precipitate in the centrifuge-tube was washed with successive small volumes of slightly alkaline distilled water (pink to phenol red) which had already been used to wash out the flask in which precipitation had occurred, and these washings were also filtered through the paper into the dish. The final volume was 20 ml. and to this 10 ml. of 97 % alcohol were added. The *p*H was brought to 7 (orange to phenol red) and 2 ml. of 10 % silver nitrate solution were added to precipitate the dicarboxylic acids present. The precipitate was allowed to stand for 5 min. to flocculate and then filtered through asbestos on a Gooch crucible. The dish was washed out with 15 ml. of 30 % alcohol and the washings were filtered through the crucible. This was repeated several times. The asbestos mat was carefully detached with a glass rod from the crucible and transferred to a glass dish where it was suspended in a few ml. of very dilute nitric acid. The sides of the crucible were washed with a little more of the acid which was added to that in the dish. The suspension was titrated with 0·01 *M* potassium thiocyanate solution, iron alum in nitric acid being used as external indicator.

Test for accuracy of method.

	1	2	3
Fumaric acid added to muscle (mg.) (0·4 g. of muscle in each of 3 cups)	3·03	2·02	0·5
Fumaric acid found (after subtraction of amount of dicarboxylic acid already in muscle)	3·04	2·00	0·51

(*b*) *Malic acid.* The trichloroacetic acid extract (remaining after ether extraction) and the titrated alcoholic solution were each evaporated down to a small volume, neutralized to litmus with NaOH and made up to 8·4 ml. with distilled water in a graduated tube. The optical rotation of the solutions was measured with sodium light and each solution was evaporated to 4 ml. 4 ml. of a 14·2 % ammonium molybdate solution and 0·4 ml. of glacial acetic acid were added, the solutions thoroughly mixed and allowed to stand in the dark for 2–3 hours [Auerbach & Kruger, 1923]. The rotation was again measured and the amount of malic acid was calculated as, under these conditions, the rotation changes 0·21° for each mg. of malic acid in the solution.

Test for accuracy of method.

	1	2	3
Malic acid added to muscle (mg.) (0·4 g. of muscle in each of 3 cups)	1·66	1·0	0·50
Increase in rotation	0·34°	0·22°	0·11°
Malic acid estimated (mg.) (after subtraction of amount already in muscle)	1·65	1·04	0·50

(*c*) *Pyruvic and oxaloacetic acids.* These acids were at first both estimated in the form of pyruvic acid, as Clift & Cook [1932, 2] showed that oxaloacetic acid and its 2:4-dinitrophenylhydrazone are decomposed quantitatively to the corresponding pyruvic acid and derivative when heated on a boiling water-bath. If the amounts of pyruvic acid found in the experiments were appreciable, a differentiation between the two acids would have been made in later work by the method of Ostern [1933] which estimates oxaloacetic acid only. The method of estimation of the pyruvic acid adopted was Szent-Györgyi's modification of the method of Case [1932] which is fully described by Needham & van Heyningen [1935].

Test for accuracy of method.

	1	2	3
Pyruvic acid added to muscle (mg.) (0·4 g. of muscle in each of 3 cups)	2·25	1·05	0·75
Pyruvic acid found (mg.) (after subtraction of any present in muscle)	2·25	1·01	0·75

(*d*) *Lactic acid.* Lactic acid was estimated by the method of Friedemann & Graeser [1933], the soluble carbohydrate being removed from the trichloroacetic acid extract by precipitation with copper sulphate and calcium carbonate.

(*e*) *Detection of the fumaric acid in the presence of succinic acid.* The dicarboxylic acid remaining after the experiments was shown to be fumaric acid by incubation with an enzyme preparation (kindly supplied by Dr D. E. Green) which distinguishes between fumaric and succinic acids. Details of this preparation and its mode of action are now in the press.

EXPERIMENTAL RESULTS.

(*a*) In the respiration experiments with unwashed tissue the contents of the manometer cups were:

	Buffer ml.	Fumarate solution ml.	Water ml.	Muscle g.
Experimental cups	2·6	0·2	0·8	0·4
Control cups	2·8	—	0·8	0·4

The oxygen uptake varied with the individual pigeons but the duplicates and controls agreed well. It appeared from the results of all experiments that, with phosphate buffer as suspension medium, the addition of 2–4 mg. of fumaric acid to 0·4 g. of minced muscle produced a stimulation of respiration, that of the muscle with added acid being always higher than that of the control from the beginning of the experiment (Fig. 1).

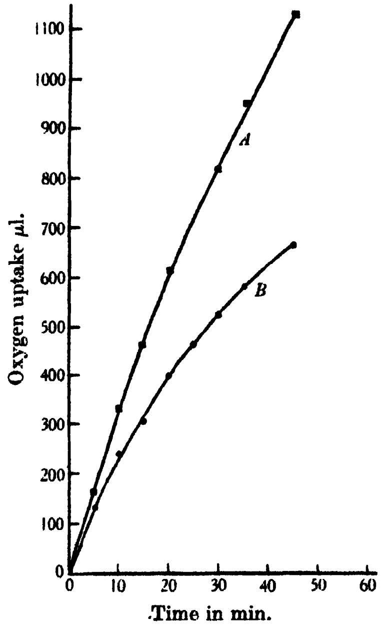

Fig. 1.

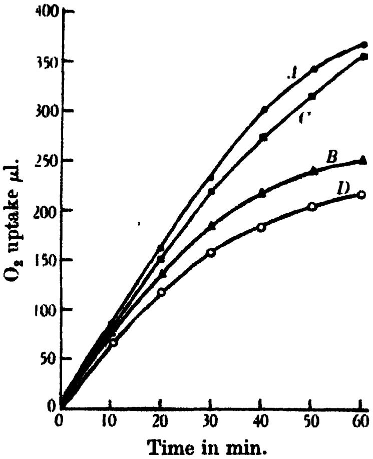

Fig. 2.

Fig. 1. O_2 uptake of minced muscle with and without added fumarate. *A*, ■—■, with added fumarate; *B*, ●—●, control without added fumarate.

Fig. 2. O_2 uptake of washed minced muscle with addition of fumarate, lactate and fumarate plus lactate. *A*, ●—●, with added fumarate; *B*, ▲—▲, with added lactate; *C*, ■—■, with added fumarate plus lactate; *D*, ○—○, control without addition.

A similar effect was observed in experiments where 2–4 mg. of succinic acid were added to 0·4 g. of muscle under the same conditions.

Estimation of the fumaric and succinic acids remaining both in the experimental muscle and in the control showed that there was always a definite disappearance of some of the fumaric acid added to the experimental muscle. Estimations of the malic acid were carried out in each experiment to see if the fumaric acid was to be found in this form. It was discovered that the amount of malic acid formed depended on the oxygenation of the tissue during the experiment, as practically no malic acid was found in samples taken from vessels filled with oxygen, whereas an appreciable quantity was found in those from vessels filled with air. When the fumaric acid equivalent of the malic acid found was added to the amount of fumarate estimated, there was still a deficit.

It was thought that the missing fumaric acid might be found in the form of the other two links of Szent-Györgyi's suggested cycle. If it had been present as succinate it would have been estimated with the fumaric acid in the silver precipitation method. The other possibility was oxaloacetic acid. It had been shown, in some early experiments in which the minced muscle was incubated aerobically with fumarate, 0·02 *M* arsenite solution, bisulphite and phosphate buffer, that a bisulphite-binding compound was formed in the flask containing added fumarate in excess of that in the flask containing control muscle. (Estimations of this bisulphite-binding compound were done by the methods of Clift & Cook [1932, 1] and of Case [1932].) Preliminary estimations of the oxaloacetic and pyruvic acids in the form of the 2:4-dinitrophenylhydrazone were therefore made on the trichloroacetic acid extracts from respiration experiments. The amounts of pyruvic acid found in the extracts from muscle with added fumarate were slightly in excess of those from the controls (of the order of 0·02 mg.), but none of them was large enough to be significant in the balance sheets.

It appears therefore that the missing fumarate was not to be found in the form of the other substances in the cycle or as pyruvic or malic acid. It is also seen, from the experiments with Green's enzyme preparation, that the remaining fumaric acid was present in its original form. The extra oxygen uptake of the tissue with added fumarate above the respiration of the control was in no case greater than the amount necessary for complete oxidation of the missing fumaric acid, but in every experiment there was still a portion of the fumaric acid untraced. The fate of this untraced fumaric acid may possibly be explained in the light of the experiments with washed tissue (see below).

Assuming that the extra oxygen was used for complete oxidation of fumaric acid, balance sheets of each experiment were drawn up as follows, the results in every case being essentially the same:

Table I.

	(1) With air			(2) With oxygen		
Experiment	(*a*) 3 cups	Control	(*b*) 3 cups	(*a*) 3 cups	Control	(*b*) 3 cups
Fumaric acid added (mg.)	5·73	—	5·73	6·26	—	6·26
Fumaric acid estimated (mg.)	1·59	0·45	1·61	2·56	0·41	2·37
"Extra fumaric acid" (above control)	1·14	—	1·16	2·15	—	1·96
Oxygen uptake (μl.)	2064	1416	2053	4192	3045	4180
Fumaric acid ≡ of "extra oxygen" (mg.)	1·12	—	1·1	1·97	—	1·96
Fumaric acid ≡ of malic acid (mg.)	1·64	—	1·65	—	—	0·12
Total "extra fumaric acid" (mg.)	3·9	—	3·91	4·12	—	4·04
Fumaric acid unaccounted for (mg.)	1·83	—	1·82	2·14	—	2·22

(*b*) In the respiration experiments with washed tissue the manometer vessels contained:

Cups	Cozymase ml.	Buffer ml.	Fumarate solution ml.	Lactate solution ml.	Water ml.	Muscle g.
(1) & (2)	0·3	2·3	0·2	0·2	0·6	0·4
(3) & (4)	0·3	2·5	0·2	—	0·6	0·4
(5) & (6)	0·3	2·5	—	0·2	0·6	0·4
(7) & (8)	0·3	2·7	—	—	0·6	0·4

The oxygen uptake was measured as before, and in every experiment it was found that the respiration with added fumarate was greater than that with added lactate (see Fig. 2).

Balance sheets were drawn up as before (Table II) and from these it can be seen that, in these concentrations, the fumaric acid is always used in preference to the lactic acid unless there is an added excess of lactic acid.

Table II.

Cups	(1) & (2)	(3) & (4)	(5) & (6)	Controls
Fumaric acid added (mg.)	4·04	4·04	—	—
Fumaric acid found (mg.)	2·10	1·99	0·44	0·19
"Extra fumaric acid" (mg.)	1·91	1·80	0·25	—
Lactic acid added (mg.)	3·76	—	3·76	—
Lactic acid found (mg.)	5·11	1·77	3·83	1·23
"Extra lactic acid" (mg.)	3·97	0·54	3·83	—
Oxygen uptake (μl.)	743	754	520	421
Fumaric acid ≡ of extra O_2 (mg.)	0·55	0·57	—	—
Fumaric acid ≡ of malic acid (mg.)	0·4	0·3	—	—
Lactic acid ≡ of extra O_2 (mg.)	—	—	0·13	—

(*c*) Experiments were also carried out in which 2 mg. of pyruvic acid were added to 0·4 g. of muscle to see if this would be oxidized as rapidly as fumaric acid and therefore constitute an intermediary product in the path of oxidation. In this concentration, however, pyruvic acid depresses the respiration of the minced muscle.

DISCUSSION.

The above results (see Tables I and II) appear to indicate that fumaric acid added in the concentrations specified is utilized as the chief substrate for oxidation by the minced muscle. There is no evidence that fumaric acid in this concentration is acting as a catalyst for transference of oxygen to some other substrate in the muscle, as the missing fumaric acid is not to be found either as succinic or oxaloacetic acid, which are the other two links postulated in the cycle. The amount of fumaric acid added in each case was slightly less than that which Szent-Györgyi considers essential for prevention of diffusion of the muscle's fumarate into the surrounding medium and for the maintenance of the cycle. The effect obtained in phosphate buffer was that of raising the respiration rather than conserving it (see Fig. 1), and this does not agree with the results of Banga [1935], but does agree with the idea that the fumaric acid is being used as substrate rather than a catalyst for preservation of respiration.

The estimation of malic acid occurring in the tissue incubated with fumaric acid appears to indicate that the amount formed depends on the conditions of incubation. In oxygen no malic acid was found, whereas in air quite appreciable quantities were detected, although these did not appear to have any fixed relationship to the amount of fumaric acid present. Szent-Györgyi *et al.*, and originally Clutterbuck [1927], found that a ratio of fumarate: malate of 1 : 3 was obtained in their experiments with muscle, but the above results indicate that this equilibrium cannot be applied indiscriminately to experiments under differing aerobic conditions. As, however, in the present case, both the fumaric and malic acids were estimated by separate methods, it was unnecessary to use the method of Szent-Györgyi *et al.* of multiplying the fumarate estimated by four to obtain a true "fumarate" value. The observation that malic acid does not accumulate under good aerobic conditions is in agreement with Needham [1927] who used succinate and obtained no accumulation of malic acid in well aerated muscle.

The slight increase in the amounts of oxaloacetic and pyruvic acids in muscle incubated with added fumaric acid was not large enough to be significant in the balance sheet, but it did indicate that the oxidation of fumaric acid might be through the path of oxaloacetic and pyruvic acids, or that the Szent-Györgyi cycle was working at a much smaller concentration of the various acids than he considers necessary in the muscle. The belief that oxaloacetic or pyruvic acid is an intermediary in the path of oxidation of fumaric acid was substantiated by the experiments of incubation of minced muscle with added fumarate in the presence of arsenite and bisulphite aerobically, when a bisulphite-binding compound was formed.

The depression of respiration by pyruvic acid in the concentration used has also been noted by other workers [Elliott, 1935], but this does not rule out pyruvic acid as an intermediary, as it is probable that the tissue is still capable of dealing continuously with small quantities of the acid as formed from precursors in the muscle.

The experiments with washed tissue and added lactate and fumarate reveal that fumaric acid in any appreciable concentration appears to spare other substrates even if these are present in equal concentration. It therefore appears probable that the fumaric acid unaccounted for in the experiments with unwashed tissue was used in preference to some other substrate oxidized by the control muscle.

SUMMARY.

1. Methods of estimation of the various dicarboxylic acids involved in these experiments were worked out.

2. The addition of 2 mg. of fumaric acid to 0·4 g. of minced pigeon breast muscle suspended in phosphate buffer (*p*H 7) causes an elevation of the respiration above that of the tissue without addition. During this increased respiration some of the added fumaric acid disappears.

3. Estimations of oxaloacetic and pyruvic acids show a very slight rise in concentration after incubation of minced muscle with fumaric acid in oxygen. This may be due to the path of oxidation of fumaric acid being through oxaloacetic or pyruvic acid.

4. Estimations of malic acid indicate that the amounts of this substance accumulating in minced muscle with added fumaric acid depend on the degree of oxygenation during incubation.

5. It therefore appears that, under the conditions of the experiments and with the concentration of fumaric acid used (which is that suggested by Szent-Györgyi *et al.* as essential for maintenance of the level of fumaric acid in muscle for correct working of the catalytic cycle), the extra oxygen uptake is in no case greater than can be accounted for by oxidation of some of the fumaric acid which disappears.

6. From experiments with washed tissue it is seen that fumaric acid is oxidized in preference to lactic acid in an equivalent concentration.

7. The above results suggest that, under these conditions, fumaric acid is being utilized as a substrate for respiration and not as a catalyst for transference of oxygen to other substrates in the muscle.

I wish to thank Dr D. M. Needham for suggesting this work and for much help and advice during its progress. I am also grateful to Dr M. Dixon for advice on practical details.

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CCLXXXIX. THE EFFECT OF HALOGEN SALTS ON SALIVARY AND PANCREATIC AMYLASE.

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(*Received 22 September 1936.*)

In a previous paper [Clifford, 1925] an account was given of the effect of some halogen salts on the rate of salivary digestion. At that time the author had not realized the important part played by salt concentration in enzyme action, and therefore the results referred to one strength of halide only. The present paper is a continuation of this work using varying concentrations of salts and both salivary and pancreatic amylases.

The importance of amount of salt in amylase action has been shown by Omari [1931] and by Ambard & Trautmann [1933] who state that amylase is fixed to starch proportionately to the amount of NaCl present. McClure [1933] again quotes a statement that the activity of the amylase of potato is increased by solutions of NaF up to 1/1·75 *M*.

Experiments were therefore undertaken to determine the effects of various halogen salts on amylolytic action.

Experimental.

The method used was based on the time taken by a digestion mixture of starch, salt and enzyme to reach the achromic point with iodine.

10 ml. of 0·5 % soluble starch solution and 2 ml. of distilled water or halogen salt of requisite strength were warmed in a test-tube to 37°. 1 ml. of amylase solution was then added, the tube inverted to mix and a stopwatch started. At intervals 5 drops of the mixture were placed in 2 ml. of iodine solution (2 ml. *N*/20 I made to 300 ml. with distilled water) until no colour change resulted.

The average time taken to reach this achromic point by a series of six tubes was taken as that for any given concentration of salt. In no series was the variation greater than 10 sec. unless the time taken was above 15 min. when differences of 30 sec. were sometimes met.

The amount of halogen salt varied so that the final concentration in the tube ranged from 0·2 *M* to 0·000008 *M*, each dilution being half the previous one.

The source of pancreatic amylase was a 0·3 % solution of commercial pancreas substance, and for ptyalin saliva was collected directly into distilled water as described by Cole [1933].

Parallel experiments were made with three separate batches of pancreas substance and three different samples of saliva.

The figures given in this paper represent the results obtained with one batch of pancreas substance; the results with the other samples were similar in all respects, as were those with ptyalin except that with the latter enzyme both accelerations and relative inhibitions were slightly less marked.

Results.

Fluorides (Fig. 1).

From Fig. 1 it can be seen that K and NH$_4$ fluorides exercise an inhibitory action on amylase activity, this being specially marked with the NH$_4$ salt. This action continues to a definite concentration below which no effect whatever is seen. The Na salt is quite inert at concentrations varying from 0·5 to 0·000008 M.

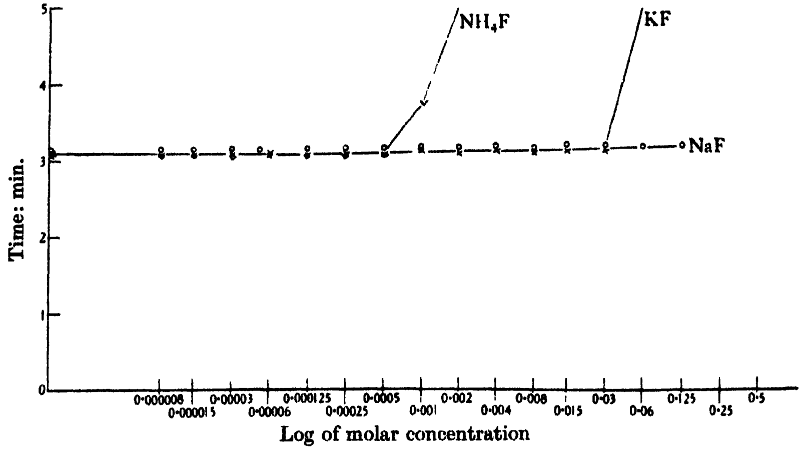

Fig. 1. Fluorides. Higher concentrations KF 10–40 min. Higher concentrations NH$_4$F no digestion in 4 hr.

o—o—o NaF. × — × — × KF. v—v—v NH$_4$F.

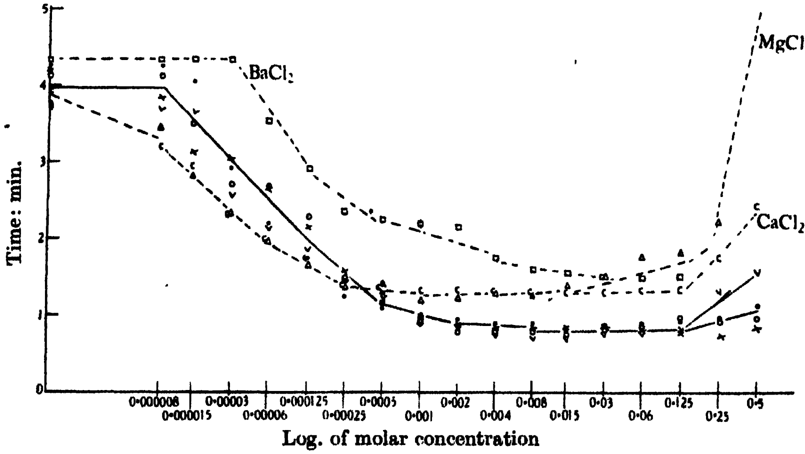

Fig. 2. Chlorides. MgCl$_2$ 0·5 M 9 min. 38 sec. for digestion.

●—●—● Li. o—o—o Na. × — × — × K. v—v—v NH$_4$.
△—△—△ Mg. c--c--c Ca. □—□—□ Ba.

Chlorides (Fig. 2).

All the chlorides investigated accelerated diastatic action, this being more marked with the Li, Na, K and NH$_4$ salts than with those of the alkaline earths.

In the case of $MgCl_2$ a concentration of 0·5 *M* actually caused inhibition, and with the Li, NH_4 and Ca salts solutions of this strength were less powerful accelerators than weaker ones. The activating power could be detected in concentrations as low as 0·00003 *M* except with $BaCl_2$ which, besides being less potent at all strengths, showed no effect below 0·0006 *M*. There was no apparent difference of acceleration with concentrations between 0·25 and 0·002 *M*, but below these the action was progressively less marked.

This is in agreement with Cole [1903] who states that solutions of NaCl between concentrations of 0·3 and 0·003 *M* are equal as accelerators of amylase activity.

Bromides (Fig. 3).

The results with these salts were similar to those with chlorides, but less marked.

Again the highest concentration of the Mg salt retarded the action of the enzyme, and the Ba salt was less potent than any other at all strengths.

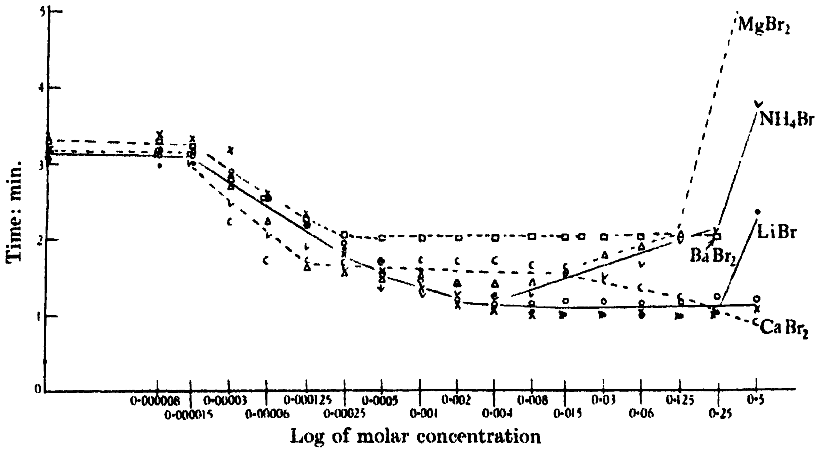

Fig. 3. Bromides. $MgBr_2$ 0·25 *M* 5 min. 23 sec. for digestion.

●—●—● Li. ○—○—○ Na. ×—×—× K. v—v—v NH_4.

△—△—△ Mg. c--c--c Ca. □—□—□ Ba.

The optimum range of concentration was between 0·125 and 0·008 *M* for the first four salts; above and below these limits acceleration was less marked. With the alkaline earths, the Mg salt gave optimum quickening between 0·03 and 0·005 *M*, the Ca salt at 0·5 *M* with slightly less activity from 0·25 to 0·006 *M*, whilst the Ba salt gave least acceleration of all the salts of this series but acted at concentrations of 0·25–0·0006 *M*.

Iodides (Fig. 4).

The results with iodides were even more varied than with fluorides.

Li, NH_4, Mg and Ca iodides at 0·5 *M* showed inhibitory actions extending over a period of hours. This inhibition could be seen at 0·06 *M* with CaI_2, at 0·03 *M* with LiI and MgI_2 and at 0·25 *M* with NH_4I.

On the other hand the iodides of Na, K and Ba hastened diastatic activity at 0·5 *M*.

At lower concentrations all the iodides hastened the digestion of starch, Li, K, Mg and Ca showed effects at 0·0005*M* whilst the Na, NH_4 and Ba salts were less potent. Again the Ba salt showed the least action.

From these results it can be seen that all halogen salts (except fluorides) quicken the rate of starch hydrolysis by salivary and pancreatic amylases, but this acceleration is a function of salt concentration. The effect is greatest and shows over the widest range of concentrations with chlorides, the next most powerful activators being the bromides.

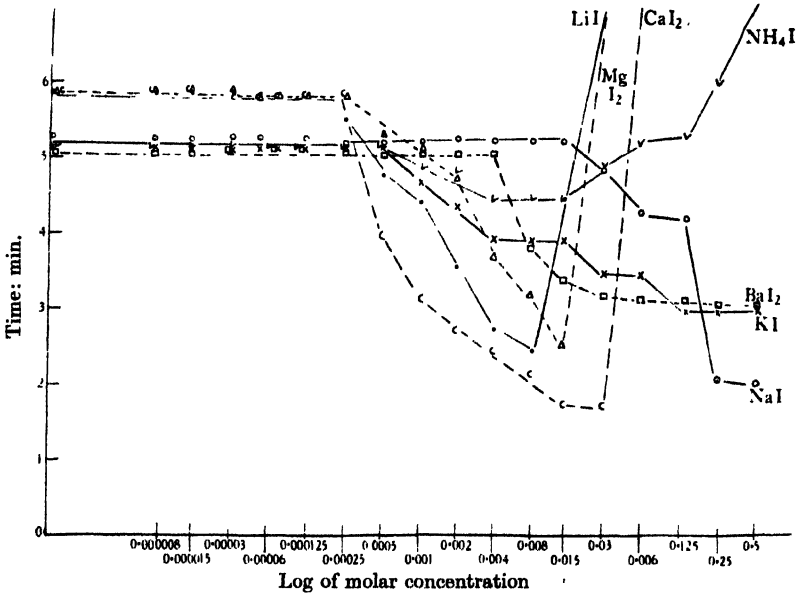

Fig. 4. Iodides. MgI_2 0·06–0·5 *M* 13 min.–2 hr. 30 min. CaI_2 0·06–0·5 *M* 12 min. 13 sec.–2 hr. 15 min. LiI, 0·3 *M* 17 min. 10 sec.; above 0·3 *M* no digestion 2 hr. 40 min. NH_4I, 0·4 *M* partial digestion 4 hrs. 30 min.

•—•—• Li. o—o—o Na. ×—×—× K. v—v—v NH_4.
△—△—△ Mg. c--c--c Ca. □—□—□ Ba.

With iodides there is still less acceleration of amylase action, and at the higher concentrations used there may be complete or relative inhibition.

Fluorides have never given any acceleration. NaF is completely inert, whilst the K and NH_4 salts inhibit at higher concentrations and are inert at lower ones.

The relative effects of halides in hastening amylase action are therefore in the order chlorides > bromides > iodides > fluorides.

The cation is not without effect since all the alkaline earth salts are less potent than those of Li, Na, K and NH_4 and the Ba salt is consistently the least potent in the series, whilst the Mg salt in the higher concentrations is more inhibitory than either the Ca or Ba salt.

SUMMARY.

1. Chlorides, bromides and iodides, of Li, Na, K, NH_4, Mg, Ca and Ba hasten the hydrolysis of starch by pancreatic and salivary amylases.

2. The relative potencies are in the order chloride > bromide > iodide > fluoride.

3. Na, K and NH_4 fluorides do not hasten amylolytic action and at higher concentrations the two latter salts inhibit.

4. Li, NH_4, Mg and Ca iodides inhibit amylase activity at higher concentrations, but accelerate at lower ones.

5. The Ba halides are less potent in their action on amylolytic activity than any other halide investigated.

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CCXC. THE ASSOCIATION OF XYLAN WITH CELLULOSE IN CERTAIN STRUCTURAL CELLULOSES.

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(*Received 30 September 1936.*)

THE cellulose of most plant materials and woods differs from cotton cellulose in many respects. In the first place, separation from other cell wall constituents can only be achieved by a more or less severe treatment, in contrast to that of the cotton hair which can readily be purified. In the second place, the product obtained, while consisting mainly of "true cellulose" as typified by that of cotton, is not exclusively so, and contains also other polysaccharides, intimately associated and tenaciously retained. For this group Hawley & Norman [1932] have suggested the name "cellulosan" implying thereby a hexosan or pentosan found with the cellulose and held in some way by it. In this paper will be described some observations on the celluloses of certain plants, mostly cereal straws, which have a high cellulosan content. Attempts have been made by chemical means to obtain information as to the relationship between the "true" cellulose and associated cellulosans. A parallel investigation, involving the X-ray examination of cellulose fibres high in cellulosan, has been carried out in conjunction with Mr W. T. Astbury and will be reported later.

The present concept of the structure of the cellulose molecule has been developed by a combination of chemical and physical methods, which have shown that the peculiar and valuable properties of cellulose are not conferred by its molecular size so much as by the state of molecular aggregation in which it ordinarily exists. While actual replacement of any of the side hydroxyls in the units of the cellulose chain would not affect the strength of the chain longitudinally, the increased separation of the chains by the substitution of larger groups for the hydroxyls would tend to weaken the fibrous structure of the aggregate by overcoming the secondary valency forces responsible for lateral stability. It is stated that nitrocellulose retains its fibrous structure better than the acetate, since in the latter wider separation is brought about. In view of these facts, the existence of lignocellulose or pectocellulose as a definite combination between oriented cellulose and lignin or pectin cannot be admitted and the possibility of direct combination between cellulose and cellulosans is similarly ruled out. The evidence to be presented is in accord with the view that the cellulosan molecules participate in the cellulose micellae and crystallites in the same way as do the individual cellulose chains. The commonest cellulosan is xylan, though in Gymnosperms mannan is characteristic. The xylan from esparto has been shown to be a 1:4-anhydroxylose, the xylose units being of the normal amylene oxide type [Hampton *et al.*, 1929]. A terminal group of *l*-arabinose has also been detected in this particular case [Haworth *et al.* 1934]. The xylan unit therefore differs from the glucose unit in cellulose only in that the terminal carbinol group is missing. Such units are of the same size and would occupy the same space longitudinally. The mannan formed in the cellulose from Gymnosperms has not been investigated, but by analogy is likely to be also a 1:4-anhydrohexose and, therefore, sterically similar in size to the cellulose and xylan with which it is so intimately associated.

The cellulosans are probably laid down with the cellulose as it is formed and participate in the oriented bundles of molecules that form the micellae of the fibres. They would be retained by secondary valency forces just as the cellulose chains are themselves stabilized in that way. Because of the absence of a projecting carbinol group, in the case of xylan at least, these forces might be expected to be less powerful than those between the cellulose molecules themselves. Some evidence will be presented later on the relative rates of removal of xylan and mannan from natural celluloses containing both. Further, the molecular size, or rather the length of the chain of sugar units, is considerably less in the case of the cellulosans than in cellulose itself. As a result of their retention by secondary valencies weaker than those between the cellulose chains and of their smaller molecular size they might be expected to be extractable, if with some difficulty. The actual form of participation of the cellulosans in the micellae is, at present, a matter of speculation. They undoubtedly form a normal integral part of the cellulosic structure of the plant cell wall and fibres, and any study of natural celluloses should include this group in as unchanged a condition as possible.

PREPARATION OF CELLULOSE.

Norman & Jenkins [1933] described a method for the determination of cellulose which could readily be adapted for large-scale preparations. Previously chlorinations for the removal of lignin have been carried out by some modification of the original procedure of Cross & Bevan [1918] employing gaseous chlorine, by which means it is impossible on a laboratory scale to treat more than a few g. at a time. By the use of dilute hypochlorite solutions quantities up to 1 kg. may be readily treated. Six or seven treatments are required for such materials as cereal straws in bulk and rather more for woods. The final washing must be continued until no trace of sulphite is detectable in the filtrate.

A. EFFECT OF HEAT ON CELLULOSE PREPARATIONS.

Cellulose preparations containing cellulosan undergo an irreversible change on oven-drying, as a result of which a fraction becomes soluble in hot water. If this be removed and the preparation again oven-dried, a further but smaller fraction can be extracted. This process can be repeated apparently indefinitely. Fig. 1 shows the losses from a sample of oven-dried wheat straw cellulose on boiling with water, the cellulose being oven-dried overnight between each

Table I. *Extraction of wheat straw cellulose, wet and oven-dried.*

Initial furfuraldehyde yield 13·88 %, equivalent to 21·59 % xylan.

Treatment	Total loss %		Loss of xylan %	
	Wet	Dry	Wet	Dry
3·0 % Na_2SO_3 2 × 20 min. hot	5·0	7·6	1·1	2·8
0·25 % NaOH 1 hour hot	11·7	19·0	3·3	6·0
1·0 % NaOH 1 hour hot	17·5	22·0	5·4	7·2
4·0 % NaOH 3 hours cold	18·8	22·5	13·2	16·4
0·25 % H_2SO_4 1 hour hot	9·4	11·7	4·7	6·2
2·5 % H_2SO_4 1 hour hot	11·9	15·6	7·7	10·4

treatment. After the fifth extraction the xylan content of the residue was determined. Whereas the aggregate loss was 18·8 % the xylan removed accounted for only 8·9 %, or about half.

The effect, therefore, is not solely concerned with the cellulosan fraction. Further, the effect of heat-drying also renders the cellulose preparations more susceptible to extracting and hydrolysing agents, as shown in Table I in which the losses from wet and dried preparations are compared. Again, the differences are not wholly due to an effect on the xylan, and must arise in part from a change in properties of a portion of the "true" cellulose fraction.

To determine whether this effect is due to the removal of water in drying or the application of heat, or both, a large batch of oat straw cellulose was subdivided and dried in different ways. These samples were then extracted by boiling with water for 15 min., and since the effect of certain initial treatments was noticed to persist after the first extraction, all the residues after drying for 16 hours at 100° were twice re-extracted with water and again dried. The results are summarized in Table II.

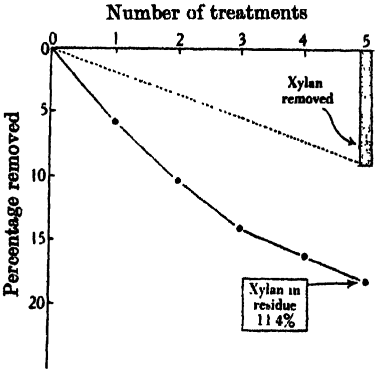

Fig. 1. Water-soluble material produced on repeated drying of wheat straw cellulose (xylan 20·3 %).

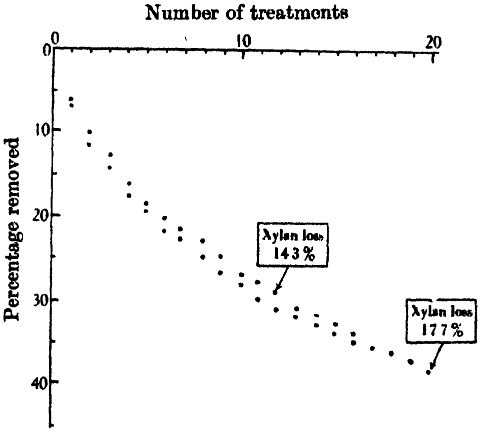

Fig. 2. Repeated extraction and drying of oat straw celluloses.

Exposure to heat seems to be the vital factor in this phenomenon. Whereas there was little difference in the material when air-dried, alcohol-dried or dried at 40°, higher temperatures enhanced the amount of water-soluble material obtainable. Air-dried or alcohol-dried samples subsequently exposed to heat were similar in properties to those directly given the same heat treatment when wet. Wet samples which were alternately rapidly frozen and thawed showed no change in properties.

Results of a similar character were obtained when extracting agents other than water, such as dilute acids and alkalis were employed. The effect of exposure to heat results in some modification of the properties of a portion of both components of isolated plant celluloses, which is manifest in increased susceptibility to extracting and hydrolysing agents. Moreover, this effect may be demonstrated repeatedly upon the same sample. The increments of water-soluble material become smaller, but are still appreciable after many treatments. In Fig. 2 the amounts removed from two samples of cellulose alternately dried and extracted are shown. Over the first twelve treatments the xylan

Table II. *Loss on water extraction of oat straw cellulose dried by various means.*

All results expressed on basis of 100 g. oven-dried material.

		Total loss		
Series	Treatment of cellulose	Extraction I	Residue from I, dried 16 hr. at 100° Extraction II	Residue from II, dried 16 hr. at 100° Extraction III
A	Wet	4·3	6·2	8·7
B	Air-dried; room temperature (moisture content 7·53 %)	4·7	6·5	9·0
C	Alcohol-dried, room temperature	5·2	7·5	9·1
D	Dried at 40°	5·3	7·9	9·9
E	Dried at 60°	6·3	9·0	11·6
F	Alcohol-dried; put in benzene, heated under vacuum at 80° for 10 hr.	6·2	7·6	9·5
G	Dried at 80°	6·6	9·3	11·3
H	Oven-dried, 100° for 8 hr.	6·8	11·6	13·6
I	Air-dried, then heated at 100° for 16 hr.	7·6	9·7	14·0
J	Alcohol-dried, then heated at 100° for 16 hr.	7·1	10·9	14·2
K	Vacuum-dried, at 100°	8·1	10·2	12·8
L	Dried at 120° for 16 hr.	8·9	11·9	13·3
M	Dried at 150° for 16 hr.	9·6	11·7	14·2

Cellulose contained initially 32·3 % xylan.

removed accounted for about half the total loss and rather less after 20 treatments, at which point 80 % of the xylan had been removed, as compared with a loss of about 25 % of the "true cellulose". The xylan or cellulosan is therefore much the more susceptible fraction. Concurrently a distinct change in physical properties was observed on repeated extraction, the cellulose residue becoming less cohesive and losing the slightly gelatinous property which caused it originally to dry to a close hard mass, being instead light and friable.

Whilst the effect of heat on plant celluloses is to cause the formation of some water-soluble material, and whilst this effect can be repeated apparently indefinitely, it must be pointed out that pure cotton cellulose does exhibit the same phenomenon, but to a much smaller extent. An unbleached cotton yarn from

Table III. *Loss on water and citric acid extraction of cotton cellulose, oat and wheat straw cellulose after drying at 100° overnight.*

Expressed on 100 g. cellulose (oven-dried basis).

	Water: 15 min.			0·5 % citric acid: 1 hr.		
Extraction	Cotton	Oat straw	Wheat straw	Cotton	Oat straw	Wheat straw
I	3·6	6·8	7·3	7·6	13·6	11·8
II	4·3	11·6	9·4	7·8	21·2	17·6
III	4·9	13·6	11·0	8·4	26·6	23·3
IV	5·2	17·1	13·0	8·7	28·8	25·1

high quality long fibre Egyptian cotton was treated with water and 0·5 % citric acid for comparison with cereal straw celluloses. The results are given in Table III.

The isolated celluloses of cereal straws, containing as they do some 20 % xylan, are clearly much more affected by heat-drying than is pure cotton cellulose entirely free from xylan. Since it is evident that the water-soluble material formed cannot be solely accounted for from the xylan, the inference is that the presence of cellulosan weakens the whole cellulosic aggregate and causes a greater degree of susceptibility to the effect of heat.

The nature of the water-soluble material.

The aqueous extract obtained on boiling an oven-dried cellulose preparation is only slightly reducing. For example, 1 g. oat straw cellulose extracted after oven-drying lost 68 mg. The extract titrated by the Shaffer-Somogyi [1933] micro-method contained reducing groups equivalent to 0·4 mg. glucose, a reducing value of 0·58 % on the material extracted. Having established that the water-soluble material produced by drying is not hydrolysed by aqueous extraction, preparations were made from various isolated celluloses. The dried cellulose was boiled with water for 15 min. and the extract, after filtration, concentrated to a small volume under reduced pressure. To this straw-coloured liquid 5 vol. of alcohol were added, the white precipitate which formed being washed with acid alcohol, dried with increasing concentrations of alcohol and finally in a desiccator over fused zinc chloride. The preparations were slightly greyish white in colour, very light and hygroscopic.

Analyses showed that the preparations contained both hexose and pentose units as would be expected and some uronic acid groups. The anhydropentose content varied from 60 to 75 % and uronic acid anhydride from 5 to 8 % according to the source. Acidic groups were titratable with $N/50$ NaOH using thymol blue as indicator. A study was made of the rate of hydrolysis of certain of these preparations, the reducing sugar formed being titrated by the Shaffer-Somogyi micro-method, with the modification that the time of heating was increased to 25 min. since the shorter period recommended for glucose did not give complete recovery of xylose. The initial reducing values before hydrolysis were low, but rather variable, from 0·17 to 3 % calculated as glucose. Hydrolysis by boiling with dilute mineral acids was rapid, but the recoveries of sugar were not very satisfactory. The shape of the hydrolysis curves did not suggest that there was a resistant portion unattacked by any of the acid strengths employed, but rather that the incompleteness of the recovery must be ascribed to destruction of sugar. It is known that high yields of xylose from xylan are difficult to obtain [Heuser & Brunner, 1922; Heuser & Jayme, 1923] and that the decomposition of pentoses during the hydrolysis of pentosans is greater than in controls of pure sugar similarly heated with acids. Since all the preparations were relatively rich in xylan, destruction of xylose probably accounted for the low sugar yields. The recovery of sugar from two preparations from oat straw cellulose is shown in Table IV.

Since a strong probability existed that these preparations were not homogeneous, an attempt was made at separation by precipitation at different alcohol concentrations. Four fractions, A, B, C, D, were obtained at 48, 65, 85 and 95 % alcohol concentrations respectively. The yields from 90 g. oat straw cellulose were A, 1·6 g.; B, 0·39 g.; C, 0·14 g. and D, 0·04 g. The furfuraldehyde yields indicated the xylan contents to be respectively 70·5, 43·4 and 52·8 %, the amount of D available being too small for any determination. The initial

Table IV. *Hydrolysis of water-soluble material from oven-dried oat straw cellulose.*

% sugar liberated expressed as apparent glucose.

Acid concentration	Time	Prep. 1 Xylan content 68·5 %	Prep. 2 Xylan content 73·4 %
5 % H_2SO_4	5 hr.	88·3	89·2
2·5 % H_2SO_4	3 hr.	82·9	89·8
1 % H_2SO_4	3 hr.	82·8	86·5
10 % oxalic	3 hr.	93·1	85·9
2·5 % H_2SO_4	1 hr. at 1 atmos.	84·2	85·6
72 % H_2SO_4	2 hr. cold, diluted to 3 % and boiled 2 hr.	79·7	81·5
3 % HNO_3	1 hr.	70·7	—

reducing value of the main fraction A was 1·09 % in terms of glucose. The hydrolysis curves obtained with 10 % oxalic acid and 0·15 *N* H_2SO_4 were similar in shape, and the recoveries of sugar again unsatisfactory.

Evidence that the main fraction A obtained at the lowest alcohol concentration was still not homogeneous was given by separation by other means. A small amount was dissolved in cold 1 % NaOH and Fehling's solution added. The blue precipitate which formed, after washing with 50 % alcohol, was suspended in alcohol containing HCl and washed free from copper. This precipitate was found to yield furfuraldehyde equivalent to a xylan content of 86 %. The yield however was small and did not represent all the xylan in the sample taken. A similar partial separation of xylan was effected by adding alcohol to the alkaline filtrate from the copper precipitation above. A fraction was obtained at an alcohol concentration of 50 % which on subsequent washing and drying was found to contain 84 % xylan. Both procedures gave a fraction richer in xylan than the original preparation, which might be held to substantiate the view that the hexosan and pentosan are not combined.

The alcoholic filtrates from the precipitation of the water-soluble substance contained a small quantity of material not precipitated even at an alcohol concentration of more than 95 %. This was obtained by distillation under reduced pressure to a very small volume which was then made up to 10 ml. The solution was golden brown in colour and strongly reducing. On hydrolysis with acid the reducing value increased only about four or five times, an indication that the molecular size of this fraction must have been small. Figures for three such residues are given in Table V.

Table V. *Reducing power of alcohol-soluble fraction.*

Expressed as mg. glucose.

	I	II	III
Direct	48·3	37·5	33·0
Hydrolysed for 1 hr. with 5 % H_2SO_4	180·0	111·0	—
Hydrolysed for 3 hr. with 2·5 % H_2SO_4	—	—	142·0

Changes in the cellulose as a result of drying.

There are few tests that can be applied to cellulose itself to obtain information bearing on the effect of heat-drying. Degradative changes are usually accompanied by an increase in the copper number as a result of the production of reducing groups. Such an increase was observed, the method employed being the Heyes [1928] modification of the Schwalbe-Braidy procedure. A small concurrent increase was found in the yield of CO_2 on distillation with 12 % HCl presumably owing to the production of uronic acid groups. It is not certain, however, that all the CO_2 given by cellulose is due to this grouping. The figures are given in Table VI.

Table VI. *Changes in copper number and CO_2 yield on drying wheat cellulose.*

Treatment of cellulose	Copper number*	CO_2 number†
Wet	0·60	0·30
Air-dried	0·75	0·43
Oven-dried, 16 hr.	0·83	0·45
Oven-dried and extracted with water twice, then air-dried	0·98	0·46
Oven-dried, 48 hr.	2·07	—

* Weight of copper reduced by 100 g. cellulose (dry).
† Yield of CO_2 on distillation of 100 g. cellulose (dry).

The changes, though small, are in the same direction as observed in the production of oxycelluloses and are, no doubt, of a similar type.

Discussion of the above observations.

This effect of heat drying of plant celluloses does not appear to have been directly recorded before, though in a brief paper by Sherrard & Blanco [1932] some experiments are given in which the same phenomenon undoubtedly occurred. They showed that from white spruce cellulose, kept wet, some material could be removed by prolonged boiling with water, but after drying the losses from the cellulose were heavier. Partial hydrolysis due to the long period of boiling was however the chief factor concerned in their experiments. From the extract, by concentration and precipitation with alcohol, a white powder was obtained, rather similar to those prepared in this work, in that it was very hygroscopic and easily hydrolysable to sugars. Experiments on the drying of wood made by Campbell & Booth [1930; 1931] showed a slight increase in the hot water-soluble fraction as a result of treatment at 100° or 105°, a fall in the Cross & Bevan cellulose content and a definite increase in alkali-solubility. These changes may be in part accounted for by the effect of heat on the cellulosic fraction.

The observations described in this paper do not admit of any ready explanation on the basis of present theories of the arrangement of cellulose molecules. The removal of water by drying is likely to modify conditions within the micellae. Shrinkage will occur and strains be set up as a result of the withdrawal of the "packing" provided by the water molecules. This disturbance might be expected to make for an increased susceptibility to the hydrolytic action of boiling water or stronger reagents. The observed effect of heat is, however, more difficult to understand. Alcohol-dried and air-dried samples subsequently exposed to a temperature of 100° were considerably affected by this further treatment from which water relationships are excluded. The effect of moderate temperatures on cellulose has been little studied. Profound changes take place on prolonged heating; indeed, it has been stated that complete carbonisation may occur in the course of a few months at 120°. Knecht [1920] has shown a rapid loss of colour and strength in cotton exposed at 93°. Whatever be the nature of the changes caused by the application of heat, plant celluloses containing cellulosans are very much more affected than is pure cotton cellulose. Both the initial loss on extraction and the further increments obtained by repeating the treatment are considerably larger than in the case of cotton. The extra amounts are only in part accounted for by the cellulosan removed. The "true" cellulose aggregate is therefore less resistant than cotton or is weakened in this respect by the presence of cellulosan molecules within the micellae. The preparations of water-soluble material obtained do not throw much light on the nature of the changes responsible for the production of this fraction. The presence of two components which are undoubtedly affected to a different extent by the treatments means that any preparation will be a mixture. The cellulosan fraction is more affected by heat treatment than is the "true" cellulose since the xylan content of the water-soluble preparations was about 70 %. It must be presumed that the effect of heat is to cause a breakage of some of the cellulose chains, and probably but not essentially a breakage of the xylan too. Evidence for chain breakage is provided by the increase in "copper number" values.

At the same time, some oxidation undoubtedly occurs with the production of uronic groupings and possibly also of terminal carboxyl groups of the gluconic type which would not be included in the estimation by CO_2 yield. Without assuming definite breakage of the cellulosic chains as a result of heat, it is not possible to account for the production of further water-soluble fractions, apparently similar, on wetting and re-exposing to heat. The water-soluble material may be supposed to consist of cellulosic fragments still of considerable though variable size and partially oxidized, mixed with cellulosan or degraded cellulosan molecules. Fractionation of the water extract was undertaken since it seemed likely that cellulosic fragments of diminishing molecular size might be precipitated by increasing alcohol concentrations and that differences in these might be evident in the rates of hydrolysis. In fact, however, the major part of the material removed precipitated at a relatively low alcohol concentration. No information exists as to the limit in number of glucose units in a chain imposed by water-solubility. A very small portion of the extract was strongly reducing and, since hydrolysis increased the reducing value only three or four times, the chain length of this fraction was probably quite short. The reducing value of the preparations was low, but cannot be taken as a reliable or even approximate index of average chain length because, owing to oxidation, the terminal groups might well be carboxylic rather than aldehydic in nature.

These observations may be of practical importance in two directions. In the first place, there is the possibility that the drying of any plant material before isolation or determination of the cellulose in it may have so changed the balance between cellulose and cellulosan that the product obtained is not truly representative. It is not certain that *in situ* and in association with other cell wall constituents such a change would occur. If it does, this is clearly a normal process in senescence and would take place during the ripening of straw, for example. Experiments to test this point are at present inconclusive, owing to the difficulty of grinding fresh tissues to as fine a state of division as dried materials. In routine work, at present, cellulose determinations are carried out on air-dried rather than oven-dried material.

A further implication of this work lies in the decomposition of cellulose by micro-organisms. The fermentation of celluloses which contain cellulosan has been little studied, the substrate in most work of this type being the pure celluloses of filter-paper or cotton. Heat treatment of a plant cellulose by producing a water-soluble fraction might expedite attack on the aggregate. If higher temperatures, possibly up to 140°, were employed, such a preliminary "cracking" might result in a more effective and rapid decomposition. This point is at present under investigation.

B. HYDROLYSIS AND EXTRACTION OF CELLULOSE PREPARATIONS.

Although most celluloses consist of two components, cellulosan and "true" cellulose, no quantitative separation has ever been achieved. The properties of the cellulosans are, with certain limitations, those usually described for the encrusting polyuronide hemicelluloses. That is to say, they are soluble in cold dilute alkalis and hydrolysed to their constituent sugars by boiling with dilute mineral acids. Limitations are conferred by reason of their association with the cellulose. The purpose of the work to be described was to investigate the removal of the cellulosan fraction of some celluloses by hydrolysis with acids and extraction with alkali.

There are few references in the literature to any previous work on this subject, indeed, only one paper, by Hawley & Fleck [1927], deals directly with the action of dilute acids on isolated cellulose. They showed that wood cellulose is hydrolysed much more readily than cotton cellulose and claimed that from softwood cellulose all the mannan and most of the pentosan might be removed with ease. The effect of dilute acids on cotton cellulose, as followed by the liberation of sugar, was determined by Wohl & Blumrich [1921] who found that the acid extract filtered free of cellulose had an increased reducing value on further boiling, indicating the presence of some compound intermediate between cellulose and glucose. A number of workers have studied the hydrolysis of wood with dilute mineral acids at ordinary temperatures and under pressure. Miller & Swanson [1925] showed that as a result of such treatment the cellulose is much reduced, a portion being lost at very low acid concentrations. This easily hydrolysable portion, amounting to about 13 % of the cellulose, was, no doubt, largely cellulosan in nature.

Only recently has it been realized that the cellulose of plant materials and woods is much less resistant to the action of acid and alkali than is cotton cellulose. Any treatment other than in neutral solution should be avoided in handling materials from which the cellulose is to be prepared or determined, in order that the integrity of the cellulose may be preserved. The nature of the attack brought about by acid and by alkali is shown in the following experiments. In view of the observations on the effect of heat-drying, all preparations were kept wet, and the treatments to be described were carried out on the wet material, due allowance being made for the moisture content.

1. *Hydrolysis.*

The effects of hydrolysis were studied by treating small samples for various times under standard conditions. The residues after thorough washing and drying were weighed and used for furfuraldehyde determinations. Reducing sugars were estimated directly in the extract using the Shaffer-Somogyi [1933] micromethod. The acid extract was also further hydrolysed by boiling again for 3 hours after adjusting the acid concentration to 4 % in each case. The reducing values of the extract both direct and after rehydrolysis are expressed as apparent glucose, it having been determined that the curve for xylose with this reagent is almost identical with that for glucose. With this information a full picture of the changes taking place on hydrolysis may be obtained. In Tables VII and VIII respectively are given the results obtained on the celluloses of oat straw and oak.

Considering first the series on oat straw cellulose given in Table VII, this cellulose was found to undergo appreciable loss with acid as dilute as 0·1 % (*N*/50) and in a period as brief as half an hour. Hydrolysis was progressive with time in this and all higher concentrations. Three important facts emerge and may

Table VII. *Hydrolysis of oat straw cellulose with acids at the boiling point. Furfuraldehyde yield = 17·82 % equivalent to xylan 27·62 %.*

All results expressed on the basis of 100 g. original cellulose.

Conc. of H_2SO_4 %	Time in hr.	Residue	Xylan in residue	Xylan lost	Hexosan lost (diff.)	Sugars in extract	Sugars on re-hydrol.	$\frac{\text{Xylan loss}}{\text{Total loss}}$ %
0·1	½	93·1	23·4	4·2	2·7	2·7	8·8	61
	2	89·3	21·4	6·3	4·4	4·2	10·2	59
	5	88·2	20·8	6·8	4·9	6·0	12·1	58
	16	84·4	17·1	10·5	5·0	10·7	13·7	67
0·25	1	87·3	19·8	7·8	4·8	4·5	9·9	67
	2	85·7	18·1	9·6	4·8	6·5	12·2	67
	3	84·6	17·5	10·1	5·3	7·4	12·5	66
	5	82·7	16·2	11·4	5·8	10·5	13·1	66
	16	79·4	15·0	12·6	8·0	14·8	14·2	66
1·0	½	85·6	17·1	10·5	3·9	5·8	11·7	73
	1	83·9	16·9	10·7	5·3	8·6	13·9	67
	2	82·8	16·3	11·3	5·9	12·0	14·6	66
	3	81·7	15·2	12·4	5·9	13·5	14·8	68
	5	81·3	14·1	13·5	5·2	15·9	16·9	72
	16	77·2	8·9	18·7	4·1	22·4	21·4	82
3·0	½	84·7	16·8	10·8	4·5	10·9	14·4	71
	1	82·8	15·2	12·4	4·7	14·2	15·8	72
	2	80·7	12·8	14·8	4·5	17·1	17·7	77
	3	79·1	11·5	16·1	4·8	18·0	18·9	77
	5	76·5	9·9	17·8	5·7	19·4	20·3	76
	16	72·0	5·6	22·0	6·0	24·9	25·5	79
5·0	½	81·6	14·4	13·2	5·1	13·3	15·4	72
	1	80·6	13·8	13·9	5·6	16 5	16·9	72
	2	78·1	11·7	16·0	5·9	19·2	20·3	73
	3	76·2	9·9	17·7	6·0	21·2	—*	75
	5	74·7	8·1	19·5	5·8	24·0	—*	77
	16	67·3	3·5	24·2	8·6	27·3	—*	74

* Not determined.

be enumerated at once. Firstly, there is no apparent break in the continuity of the reaction with any of the concentrations of acid employed. If the effect produced on the cellulose in any selected time is plotted against the acid concentrations smooth curves are obtained, giving no indication of a fraction or fractions particularly susceptible to removal. Secondly, in no case did the xylan removed account for the whole of the loss sustained by the cellulose. There was evidently a concurrent extraction of hexosan, which presumably has to be considered as an attack on the true cellulose, though this point will be discussed more fully later. Thirdly, the reducing values of the filtered extracts obtained with all the lower acid concentrations were such as to indicate that the material removed had not undergone complete hydrolysis to reducing sugars. On increasing the acid concentration of the filtered extract to 4 % in those cases in which it was originally less than this, on further boiling, the reducing values increased until the apparent sugar content was approximately equivalent to the total loss in the natural cellulose. The agreements were not very precise, no doubt owing to the instability of xylose in dilute mineral acid as referred to in the previous section.

Reviewing the series as a whole, the xylan may be said to be fairly readily removable by dilute acids, the maximum loss of 87 % of the total xylan being achieved in 16 hours with 5 % acid. With lower concentrations and in shorter periods the amount was almost proportionately less. The same, however, is not

Table VIII. *Hydrolysis of oak cellulose with acids. Furfuraldehyde yield = 15·93 % equivalent to xylan 24·69 %. Lignin = 0·79 %.*

All results expressed on the basis of 100 g. original cellulose.

Conc. of H_2SO_4 %	Time in hr.	Residue	Xylan in residue	Xylan lost	Hexosan lost (diff.)	Sugars in extract	Sugars on re-hydrol.	$\frac{\text{Xylan loss}}{\text{Total loss}}$ %
0·1	½	92·3	20·2	4·5	3·1	0·5	1·9	59
	1	90·1	19·8	4·9	4·9	0·9	5·1	50
	2	88·5	18·8	5·9	5·6	1·7	6·8	51
	3	88·0	18·0	6·7	5·3	2·3	7·4	55
	5	86·1	17·7	7·0	6·8	3·6	8·4	50
	16	83·9	16·0	8·6	7·5	8·5	11·2	53
0·25	½	90·3	20·1	4·6	5·1	1·0	5·1	48
	1	89·7	19·1	5·6	4·7	2·0	6·5	54
	2	87·8	17·9	6·7	5·4	3·5	8·3	55
	3	86·1	17·6	7·0	6·8	5·8	9·9	50
	5	85·4	17·3	7·4	7·2	8·0	10·5	51
	16	81·1	12·9	11·8	7·1	12·9	11·6	59
1·0	½	88·1	17·5	7·2	4·7	3·0	7·8	61
	1	87·2	17·3	7·4	5·4	5·9	8·9	58
	2	84·9	16·1	8·6	6·6	9·1	11·7	57
	3	83·7	15·4	9·3	7·0	11·1	13·1	57
	5	80·8	13·4	11·3	7·9	14·9	15·2	59
	16	74·2	8·8	15·9	9·9	19·3	—*	62
3·0	½	86·7	16·4	8·3	5·0	7·9	10·6	62
	1	84·4	15·0	9·6	6·0	12·1	12·4	62
	2	81·1	13·0	11·7	7·2	15·1	16·3	62
	3	77·8	10·9	13·8	8·4	16·4	16·4	62
	5	74·7	8·5	16·2	9·1	20·2	—*	64
	16	69·3	4·2	20·5	10·2	24·6	—	67
5·0	½	84·5	14·8	9·8	5·7	11·2	11·0	63
	1	79·9	12·5	12·2	7·8	15·5	16·2	61
	2	76·9	10·1	14·6	8·5	18·4	18·3	63
	3	75·4	8·9	15·8	8·8	22·1	—*	64
	5	71·3	6·4	18·3	10·4	23·5	—	64
	16	64·9	2·8	21·9	13·2	25·5	—	62
10	½	76·2	9·9	14·8	9·0	10·8	—	62
	1	73·6	8·6	16·1	10·3	13·1	—	61
	2	68·8	5·8	18·8	12·4	15·0	—	60
	3	66·8	4·4	20·3	16·5	16·5	—	61
	5	60·2	3·1	21·6	18·2	16·5	—	55
	16	57·5	1·3	23·4	19·1	16·5	—	55

* Not determined.

true of the hexosan removed. The results suggest the presence of a little over 5 % of hexosan material removable from the cellulose by acid. On prolonged boiling this amount was somewhat increased. The hexosan does not show the same progressive removal as the xylan and the ratio of xylan loss to total loss increased with time except in the case of very dilute acids when it remained more or less constant. The material removed had not undergone complete hydrolysis to reducing sugars, though hydrolysis must have proceeded to a very considerable extent, for in only one experiment was the reducing value as much as doubled on rehydrolysis of the extract. The loss of xylan and hexosan must be ascribed largely to hydrolysis and not simply to extraction.

The results on oak cellulose given in Table VIII lead to similar conclusions. Some differences in degree are discernible and these are manifestations of the individuality of the cellulose. The xylan was rather less readily removed by very dilute acids, and whereas in oat cellulose about 5 % hexosan was removed concurrently with the xylan, that fraction of the oak cellulose was more susceptible, and seemed to be progressively attacked. With low concentrations of acid, the hexosan removed was almost equal to the xylan, the ratio of xylan lost to total loss being about 50 %. With higher concentrations this ratio increased but was not so high as that given by the oat cellulose. In another respect, too, differences were noted between the two samples of cellulose, the filtered acid extracts obtained being less completely hydrolysed in most cases.

Attempts were made to carry out a similar series of hydrolyses on a softwood cellulose, and although the removal of the xylan component could be followed accurately, it was not possible to distinguish in the dilute extracts between the mannose from the cellulosan and the other hexosan material presumably from the cellulose.

2. *Extraction.*

The effect of treatment of some plant celluloses with alkali was studied similarly by heating small samples for various times under standard conditions. Special precautions were needed in washing, to ensure the removal of all alkali.

Table IX. *Extraction of celluloses with boiling NaOH solutions.*

All results expressed on the basis of 100 g. original oven-dried cellulose.

Cellulose and xylan content	Conc. of NaOH %	Time	Residue	Xylan in residue	Xylan removed	Hexosan removed (diff.)	Xylan loss / Total loss %
Oak (24·69)	0·1	10 min.	92·5	20·5	4·2	3·3	56
		1 hr.	89·4	18·8	5·9	4·7	56
		5 hr.	85·0	17·6	7·1	7·8	47
	1·0	10 min.	87·9	17·5	7·2	4·9	59
		1 hr.	82·2	16·6	8·1	9·7	45
		5 hr.	76·7	15·1	9·6	13·7	41
	4·0	10 min.	76·1	10·5	14·2	9·7	59
		1 hr.	72·3	8·6	16·1	11·6	58
		5 hr.	71·1	7·9	16·8	12·1	58
Wheat straw (21·52)	0·1	10 min.	94·7	18·5	3·0	2·3	57
		1 hr.	93·3	17·8	3·7	3·0	55
		5 hr.	87·4	15·3	6·3	6·3	50
	1·0	10 min.	90·7	16·3	5·2	4·1	56
		1 hr.	80·9	15·3	6·2	12·9	33
		5 hr.	66·0	8·2	13·3	20·7	39
	4·0	10 min.	80·1	10·4	11·1	8·8	56
		1 hr.	68·6	7·1	14·4	17·0	46
		5 hr.	62·9	7·0	14·5	22·6	39
Barley straw (24·04)	0·1	10 min.	84·1	14·9	9·2	6·7	58
		1 hr.	80·6	12·8	11·3	8·1	58
		5 hr.	76·5	10·4	13·6	9·9	58
	1·0	10 min.	78·9	11·6	12·4	8·7	59
		1 hr.	73·4	9·7	14·3	12·3	54
		5 hr.	68·1	9·1	15·0	16·9	47
	4·0	10 min.	72·2	6·3	17·8	10·0	64
		1 hr.	67·0	6·2	17·9	15·1	54
		5 hr.	62·8	5·6	18·4	18·8	50
Jute (14·73)	0·1	10 min.	95·1	12·2	2·5	2·4	51
		1 hr.	94·2	12·4	2·3	3·5	39
		5 hr.	90·1	11·3	3·4	6·5	34
	1·0	10 min.	93·1	12·3	2·4	4·4	36
		1 hr.	88·7	10·8	3·9	7·4	35
		5 hr.	85·3	9·9	4·8	9·9	33
	4·0	10 min.	85·7	8·6	6·1	8·2	43
		1 hr.	81·0	7·5	7·2	11·8	38
		5 hr.	80·2	7·2	7·5	12·3	38

Various concentrations of NaOH from $N/50$ to $2\cdot5\ N$ were employed but results are presented only for 0·1, 1·0 and 4·0 % each at 10 min., 1 hour and 5 hours, since these conditions adequately cover the removal of the major part of the associated cellulosan. The celluloses studied included one from a hardwood, oak, two from cereal straws, barley and wheat, and one from a fibre plant, jute, the figures for all of which are given in Table IX. Alkali as dilute as 0·1 % brought about a considerable removal of xylan even in 10 min. from all celluloses, that of barley straw being particularly susceptible. The xylan was uniformly accompanied by some hexosan. With each concentration of alkali, long boiling removed hexosan at a greater rate than xylan, as a result of which the ratio of xylan loss to total loss falls with time. Not unconnected with this observation is the fact that in effecting the same total loss a higher concentration of alkali for a short period will remove more xylan than a lower concentration for a longer period. This is well shown in the oak series, in which the residue after extracting oak cellulose for 10 min. with 4·0 % acid was 76·1 % and that with 1·0 % acid for 5 hours 76·7 %. The xylan content of the former residue was, however, 10·5 % and of the latter 15·1 %. It follows that in the preparation of xylan from cellulose, a purer extract is likely to be obtained by the use of a strong alkali solution for a short period than by prolonged boiling with a more dilute solution.

The celluloses from the four different sources showed certain distinctive differences. The jute cellulose was more resistant to extraction than any of the other samples, and the losses from jute were due more to the removal of hexosan material than xylan. The celluloses from the cereal straws provided a distinct contrast, that from barley being much more susceptible to dilute alkali than that from wheat. In proportion, however, hexosan was more easily removed from wheat, and the ratios of xylan loss to total loss are consequently uniformly lower in the wheat series. Whether such differences are characteristic of these straws has not yet been determined. The results on oak cellulose provide the basis for a comparison of the effects of hydrolysis [Table VIII] and extraction. Prolonged boiling with acids produced a progressive attack on both components, that on the xylan increasing only slightly with time. As a result, on continued hydrolysis the ratio of xylan loss to total loss does not change appreciably. In alkaline extraction, on the contrary, the removal of hexosan increased faster than the removal of xylan with the result that this ratio falls.

Discussion of hydrolysis and extraction.

Treatment of a plant cellulose with hot dilute acids or alkalis results in the removal of cellulosan to an extent which depends on the concentration and the time. Concurrently, however, there is a removal of hexosan material often to an extent considerably greater than that of cellulosan. In no case, with hot reagents, is cellulosan removed unaccompanied by hexosan. Pure cotton cellulose with dilute acids and alkalis is known to undergo only very small losses under similar conditions. There is therefore in the celluloses from woods and plant materials a hexosan fraction far more susceptible than anything in cotton. The nature of this fraction and its relationship to true cellulose on the one hand and cellulosan on the other are not yet known. The existence of such a fraction has, of course, been recognized for many years, and in the evaluation of pulps a procedure is ordinarily adopted to exclude this less resistant material. By treatment in the cold with 17·8 % NaOH, a residue is obtained termed α-cellulose, which, whilst not usually free from xylan, is taken as representing the true resistant fraction available for manufacturing purposes. Bell [1932] has shown, in a study of the methylation of this fraction, that wood celluloses do not display uniformity as individuals. The alkaline extract, if acidified, gives a precipitate known as β-cellulose. The larger part of the material extracted is not however precipitated on acidification, and it is termed γ-cellulose, the amount being obtained by difference. These fractions have never been critically investigated. It is unlikely that the proportions of α-, β-, γ-cellulose have any absolute significance since their isolation rests on a purely arbitrary procedure. The β- and γ-fractions presumably include the material removed by dilute acids and alkalis as described in this paper.

From these experiments certain limited deductions as to the nature of this less resistant hexosan can be made. It is apparently as easily removed by dilute alkalis as is the cellulosan and further is readily hydrolysed by dilute acids to give reducing sugars. True cellulose is little affected by dilute alkali or acids. The resistance of true cellulose to hydrolysis by acids is unusual, even amongst polysaccharides, and has never been satisfactorily explained. This property is presumably connected with the immense chain length of the cellulose molecule, but not with its micellar arrangement, for regenerated celluloses, of inferior organized structure, are not markedly more susceptible to acid hydrolysis. The inference is therefore that this less resistant hexosan fraction of such celluloses is of much less molecular size, and possibly of the same order or chain length as the associated xylan, the properties of which are virtually indistinguishable. This hexosan fraction might perhaps be more properly regarded as a cellulosan, instead of as a portion of the true cellulose, in which case the cellulosan of such materials as described above would have two components, a xylan and a glucosan, and in this be analogous to the cellulosan of the Gymnosperms, which consists of mannan and xylan. Such a distinction is however one of nomenclature only, and does not explain the nature of the material. The mechanism which produces the celluloses of plant materials and woods seems to be less perfect in its achievement than that of the seed hairs of the cotton plant. In the latter, the fibres are composed of molecules of great chain length and of the same general order of magnitude, so that the properties are uniform. In the former, molecules considerably shorter in length appear to be laid down with the longer true cellulose chains. These shorter molecules would comprise the less resistant hexosan material removed concurrently with the xylan fraction.

C. THE EFFECT OF DESTROYING MOLECULAR ORIENTATION.

The physical and mechanical properties of cellulose depend not only on the nature of the molecule, but on the state of aggregation of the molecular units. The fact that the molecules are oriented in one direction and stabilized laterally by secondary valency forces gives longitudinal strength. If cellulose be dissolved in one of the suitable solvents and subsequently precipitated, this regularity of arrangement is lost, and mechanical strength largely disappears. If cellulosans are present and are retained by secondary valency forces, the effects of disturbing the regularity of arrangement in such a cellulose should be an increased availability of the cellulosan fraction owing to the extensive reduction of forces operating laterally. This phenomenon has been observed in the case of the cellulosan of wheat straw cellulose. Two methods of solution were employed, in cuprammonium solution and by formation of the xanthate with CS_2 and alkali. A large preparation of the cellulose from wheat straw was made and, while wet, was divided into three portions. In view of observations on the effect of heat drying, it was decided to make comparisons of the regenerated cellulose both in a wet and oven-dried condition. One third of the cellulose was dissolved in cuprammonium solution by prolonged shaking, filtered free of lumps which were broken up in a mortar until smooth, and the combined cellulose solution reprecipitated by pouring into a large volume of water containing more than sufficient HCl to neutralize all the ammonia present. The gelatinous precipitate obtained was washed by decantation and on a filter till free from copper. Half the product was then dried overnight at 100° and the remainder kept wet. The portion of the original cellulose for solution as a xanthate was treated with water and alkali to give a concentration of 17·5 % NaOH. CS_2 was stirred in, and the cellulose went slowly into solution to give a light yellow opaque liquid. It was diluted with 10 % alkali and kept overnight, giving then a clear red-brown viscous solution. The cellulose from this was regenerated by precipitation in a large bath of dilute H_2SO_4 and Na_2SO_4, a slow stream of the xanthate being run in from a tube of about 0·4 cm. diameter. In this way the cellulose was obtained in spongy irregular strings. After very thorough washing the product was divided into two portions, one being oven-dried and the other kept wet. The third portion of the original cellulose for use untreated as a control was similarly divided. In this way, six samples were obtained, untreated cellulose, wet and dried, cuprammonium cellulose, wet and dried and xanthate cellulose, wet and dried.[1] For comparison with these, similar series were prepared from two other celluloses not containing any cellulosan. One was a high-grade unbleached cotton yarn and the other filter-paper (Whatman No. 1). The origin of the cellulose of the latter was presumably wood, all the cellulosan having been removed by drastic acid and alkali treatment.

These samples were each given a number of treatments selected as a result of previous work for their suitability in providing information on the susceptibility of the cellulosan fraction. The treatments were:

$$\left.\begin{array}{ll}(a) & 0\cdot25\,\%\ \text{NaOH}\\ (b) & 1\cdot0\,\%\ \text{NaOH}\\ (c) & 0\cdot25\,\%\ H_2SO_4\end{array}\right\}\ \text{boiled for 1 hour.}$$

(*d*) Water: 2 boils for 20 min. each.
(*e*) 3 % sulphite: 2 boils for 20 min. each.
(*f*) 4·0 % NaOH: standing for 1 hour at room temperature.

The furfuraldehyde yield of the residue was determined on all wheat cellulose samples, both untreated and regenerated, and the reducing value of the extract from the two acid treatments.

The regenerated wheat celluloses from the cuprammonium and xanthate treatments were not identical in composition with the untreated wheat cellulose. In each case the basis of the solvent is an alkali, in the one ammonia (sp. gr. 0·920), and in the NaOH initially 17·5 % and later reduced to 13 %, as a result of which some of the xylan becomes extracted, and though reprecipitated on acidification is in a very fine condition and is carried away in the repeated washing that is necessary. This is particularly true of the cuprammonium product which reprecipitates in a highly dispersed condition. As a result, the xylan content of the xanthate product from wheat was 15 % and that of the cuprammonium rather less than 10 %, whilst the original cellulose contained 21·5 %. Such differences in xylan content might have invalidated the results had not the magnitude of the changes been great. The results of the extractions of wheat cellulose and its regenerated products are given in Table X, and those for cotton cellulose and filter-paper in Table XI. The results are expressed on 100 g. of the

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[1] I am much indebted to my colleague Dr S. H. Jenkins for his assistance in the preparation and reprecipitation of the cuprammonium and xanthate celluloses used in this work.

Table X. *Extraction of wheat cellulose and regenerated celluloses.*

All results except $\frac{\text{xylan loss}}{\text{total loss}}$ expressed on 100 g. of the cellulose stated (oven-dried basis).

Treatment	Residue	Xylan in residue	Xylan lost	Hexosan lost	$\frac{\text{Xylan loss}}{\text{Total loss}}$	Residue	Xylan in residue	Xylan lost	Hexosan lost	$\frac{\text{Xylan loss}}{\text{Total loss}}$	Residue	Xylan in residue	Xylan lost	Hexosan lost	$\frac{\text{Xylan loss}}{\text{Total loss}}$
	Wet: untreated. Xylan = 21·52 %					Wet: cuprammonium. Xylan = 9·53 %					Wet: xanthate. Xylan = 15·03 %				
Water	95·4	20·4	1·1	3·5	23	87·5	2·0	7·5	5·0	60	85·9	4·1	12·0	2·1	85
0·25 % NaOH	88·3	18·3	3·2	8·5	28	85·2	2·1	7·4	7·4	50	82·8	3·1	11·9	5·3	69
1 % NaOH	82·5	16·2	5·3	12·2	30	83·4	1·1	8·4	8·1	51	78·2	1·6	13·5	9·3	59
0·25 % H_2SO_4	90·6	16·7	4·8	4·6	51	87·6	1·4	8·1	4·3	65	84·2	4·1	11·0	4·8	69
2·5 % H_2SO_4	88·1	13·8	7·7	4·2	64	84·2	1·2	8·3	7·4	53	79·4	1·7	13·3	7·3	65
3 % sulphite	95·0	20·5	1·0	4·0	21	88·0	2·8	6·7	5·3	56	85·3	4·4	10·6	4·1	72
4 % cold NaOH	81·2	8·3	13·2	5·6	70	81·4	0·4	9·1	9·5	49	81·6	1·1	13·9	4·4	76
	Dry: untreated					Dry: cuprammonium					Dry: xanthate				
Water	96·7	19·6	1·9	1·4	59	86·4	3·4	6·1	7·5	45	87·3	4·0	11·0	1·7	87
0·25 % NaOH	81·0	15·5	6·0	13·0	31	80·0	2·8	6·7	13·3	33	81·9	3·0	12·0	6·1	66
1 % NaOH	78·0	14·4	7·1	14·8	32	76·0	1·7	7·8	16·1	32	78·2	1·5	13·5	8·3	62
0·25 % H_2SO_4	88·3	15·4	6·1	5·6	52	86·8	3·6	5·9	7·3	44	84·2	2·9	12·1	3·6	77
2·5 % H_2SO_4	84·4	11·2	10·3	5·3	66	77·9	2·5	7·1	15·0	32	78·7	1·3	13·7	7·6	64
3 % sulphite	92·4	18·8	2·8	4·8	36	84·8	4·7	4·8	10·4	31	86·2	4·3	10·7	3·1	78
4 % cold NaOH	77·5	5·2	16·3	6·2	73	79·2	1·1	8·4	12·4	40	81·5	1·0	14·0	4·5	76

cellulose concerned, and because of the losses of xylan in the course of preparation of the cuprammonium and xanthate products are not directly comparable with one another. Instead, the relative effects of the different treatments and the nature of the materials removed by those treatments must be compared.

Table XI. *Extraction of cotton cellulose and filter-paper and their regenerated products.*

Results expressed on 100 g. cellulose (oven-dried basis).

	Cotton cellulose						Filter-paper		
	Untreated		Cuprammonium		Xanthate				
Treatment	Air-dried	Oven-dried	Wet*	Oven-dried	Wet	Oven-dried	Untr. air-dried	Cupr. wet	Xanth. wet
0·25% NaOH	94·2	92·0	89·0	92·9	97·3	95·6	97·1	96·8	95·1
1% NaOH	94·1	91·1	88·7	91·8	96·6	92·9	95·4	94·9	95·1
0·25% H_2SO_4	94·4	93·2	87·3	95·5	98·4	93·3	97·8	98·2	95·2
2·5% H_2SO_4	93·8	91·8	83·1	91·0	95·5	92·9	96·3	92·4	93·5
Water	98·3	95·1	93·9	97·3	98·6	95·9	98·8	99·1	95·8
3% sulphite	97·0	93·0	89·9	95·2	98·0	93·3	97·4	97·3	93·8
4% cold NaOH	96·5	95·3	96·6	96·3	98·7	96·8	97·6	97·3	95·2

* This sample was very gelatinous and became highly dispersed on subsequent treatment, so that some loss occurred on filtration.

The results on the wet xanthate-regenerated cellulose are more striking than those on the cuprammonium cellulose because less xylan was lost in the method of preparation. The differences between the various extracting and hydrolysing agents were smoothed out as a result of regeneration in both cases, so that hot water removed almost as much xylan as any other reagent, in marked contrast to its action on the untreated cellulose (Fig. 3). No equivalent increase in availability of hexosan material occurred. This is in accord with the results obtained on the celluloses not containing cellulosan given in Table XI. Neither filter-paper nor cotton cellulose on solution and reprecipitation showed any significant change in susceptibility to these various treatments.

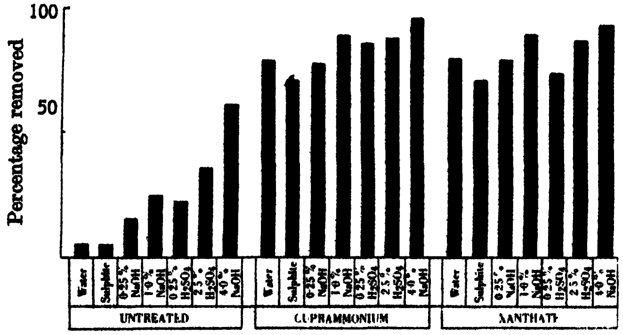

Fig. 3. Removal of xylan from untreated and regenerated cellulose.

The dried samples were very similar in properties to the wet, save that superimposed upon the changes brought about by solution and reprecipitation was also the effect of exposure to heat previously described. This appeared to be smaller in the regenerated celluloses than in the untreated material.

DISCUSSION.

It is believed that the cellulosan fraction of natural celluloses is retained by secondary valency forces. The association is, however, stronger than this type of linkage would normally provide. Additional stability is provided by the orientation of the cellulose molecular chains and their arrangement in micellae. If it is presumed that the cellulosan molecules are similarly oriented and distributed throughout the micellae, not necessarily only on the surface, then such an aggregate might be expected to have the properties of the natural celluloses as shown in this paper. A portion of the cellulosan may very readily be extracted with alkali in the cold, yet even prolonged heating with alkali fails to effect a complete extraction. This has long been a matter of comment. Heuser & Haug [1918], investigating a cereal straw cellulose, showed that many extractions with hot 6 % NaOH resulted in a loss of only 80 % of the xylan, and that even after drastic treatment under pressure at 140°–150°, 5–6 % of the xylan remained. Similar results were obtained with the wheat cellulose used in this work. The xylan content, initially 21·5 %. was reduced only to 5 % by prolonged boiling with 10 % NaOH, a loss of less than 80 %. The hydrolytic effect of acid treatment is capable of removing this resistant fraction easily, and 3 % H_2SO_4 at 100° for one hour eliminated this 5 % residue. The distribution of xylan molecules through the micellae would account for such differences in extraction.

On the solution of the cellulose and subsequent reprecipitation the organized arrangement is largely destroyed, and the secondary valency forces between the cellulose and xylan molecules are no longer operative. As a result, the xylan becomes easily removable by reagents which previously had little effect upon it. In the untreated and oriented condition about 5 % of the xylan only was water-soluble. After modification so that orientation was impaired nearly 80 % of the xylan was removed under the same conditions. Cold 4 % alkali then extracted well over 90 %, a result that could not be achieved on the untreated material, even by prolonged boiling with alkali of higher concentration. No significant increase in the susceptibility of any hexosan material occurred, this change in properties being confined to the xylan.

SUMMARY.

1. The cellulose of most plants and woods differs from that of cotton in containing associated polysaccharides, known as cellulosans, which are tenaciously retained and must be considered as an integral part of the cellulose aggregate.

2. Heat-drying produces some change in the properties of both components of such a cellulose, which is manifest in an increased availability to extracting and hydrolysing agents.

3. The effect of heat treatment may be observed repeatedly on the same sample and must involve breakage of the cellulose chains, though the xylan fraction is affected to a much greater extent.

4. Preparations of the water-soluble material produced as a result of heat-treatment are mixtures which can be partially separated to give a portion of higher xylan content. Some oxidation undoubtedly occurs, and uronic groupings are present.

5. The xylan may be removed from celluloses by treatment with either acid or alkali, but a concurrent loss of hexosan material takes place in all cases.

6. In acid hydrolysis there is no apparent break in the continuity of the reaction. The reducing value of dilute acid extracts indicates that the material removed is not completely hydrolysed to reducing sugars.

7. Continued boiling with alkali removes hexosan at a greater rate than xylan, and in effecting the same total loss a higher concentration of alkali in a short period extracts more xylan than a lower concentration for a longer period.

8. Plant celluloses show considerable differences of behaviour towards hydrolytic and extracting agents and reveal distinct individualities.

9. By solution and reprecipitation of a cereal cellulose, the organized molecular structure may be destroyed, with the result that the xylan, which was initially extracted only to a small extent by water and dilute alkali, becomes almost completely soluble. No equivalent change in the properties of the hexosan material occurs.

10. These observations are in accord with the view that the cellulosan fraction of the cellulosic aggregate of plant materials and woods is oriented and participates in the micellae, being retained by secondary valency forces identical with those which obtain between parallel cellulose chains in pure cotton cellulose.

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CCXCI. THE INFLUENCE OF CONCENTRATED POTASSIUM THIOCYANATE SOLUTIONS ON THE STRUCTURE AND THE VOLUME OF THE VITREOUS BODY.

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In an earlier paper on the vitreous body [1934] I demonstrated ultramicroscopic changes in its structure after treatment with KCNS solutions in N and higher concentrations. In these solutions the ultramicroscopic aspect of the fibrils became hazier in comparison with that of the fibrils of the fresh vitreous. The same phenomenon could be observed under the action of concentrated KI solutions and in my opinion is due to increased hydration of the fibrils. Such a lyotropic influence of concentrated KCNS and KI solutions on hydrophylic colloids is well known. The fact that salt solutions with a dehydrating action (for example K_2SO_4 solutions) caused an opposite change of the ultramicroscopic aspect, lent support to the view that in the case of KCNS the changes were due to increased hydration of the fibrils. After treatment with concentrated K_2SO_4 solutions the fibrillar pattern became much more accentuated than in the normal fresh vitreous as would be expected from dehydration of the fibrils.

In $2N$ and stronger KCNS solutions the vitreous body undergoes a rapid shrinkage to a minute fraction of its original volume. This decrease could be explained in the sense that the increase of hydration under the action of KCNS must in the last resort lead to peptization of the fibrils and accordingly to progressive solution of the framework of the vitreous.

Moreover it could be demonstrated [1935] that the vitreous never showed an increase in volume in more concentrated KCNS solutions. On the contrary, at all concentrations between 0·2 and 0·3 N there was a diminution proportional to the concentration (Fig. 1). These experiments showed therefore that increase in hydration of the fibrils does not cause increase in volume of the isolated vitreous.

This fact was important in view of later investigations [1935] in which I observed an extensive increase in volume of the vitreous after removal of the salts which are normally present. This change in volume was not due to increased hydration of the fibrils but was apparently connected with an augmentation of the capillary-electric charge of the fibrils. This increased capillary-electric activity causes greater mutual repulsion of the fibrils which leads to enlargement of the interfibrillar spaces and causes more capillary water to be taken up, so that the volume increases.

In a recent paper Duke-Elder & Davson [1935] questioned my interpretation of the shrinkage of the vitreous in concentrated KCNS solutions and expressed the view that the diminution in volume was not due to solution as indicated above but must on the contrary be regarded as the result of dehydration of the vitreous.

This criticism caused me to make further investigations of the influence of KCNS solutions on the vitreous and to make identical examinations of some other salts for comparison. A brief report of the results of these experiments is given below. It will be shown that the action of KCNS on the vitreous is quite different from those of other salts.

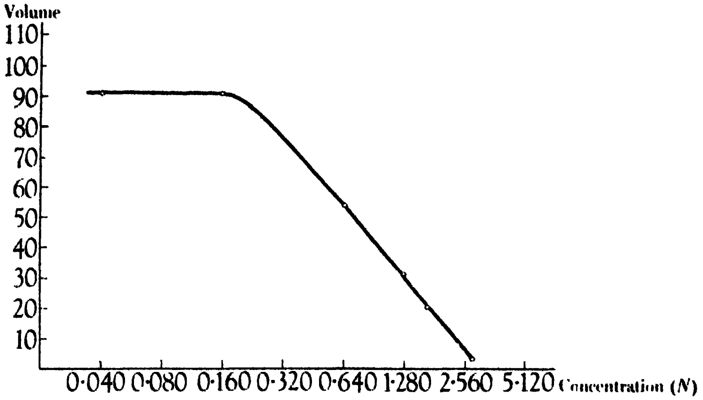

Fig. 1. Influence of KCNS solutions on the volume of the vitreous body.

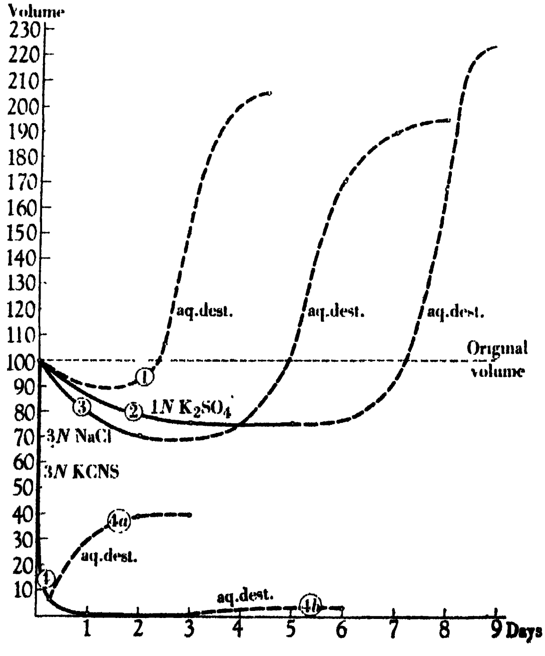

Fig. 2. —— Movement of the volume of the vitreous body in different salt solutions; – – – movement of the volume in distilled water.

Exp. 1. Some fresh vitreous were placed in distilled water which was renewed every 24 hours. After about 3 days the beginning of an increase in volume was to be observed. The maximum volume was reached after 5–6 days, and amounted to 200 % (Fig. 2, curve 1). As mentioned above the increase is connected with an increase in capillary-electric charge of the fibrils after the removal of the salts [see Goedbloed, 1935].

Exp. 2. Some other vitreous were then placed in N K_2SO_4 solutions. As is well known such a solution discharges hydrophylic colloids completely and acts moreover in a dehydrating manner. In this saline solution the vitreous decreased in volume after 5 days to 75 % of the original volume (Fig. 2, curve 2). Thereupon the vitreous were put in distilled water which was several times renewed. By this treatment the salt was removed and after some days an increase in volume was observed to a maximum of 225 %. This experiment proved that the colloid-chemical changes of the vitreous after the preceding treatment with N K_2SO_4 are completely reversible. The final volume reached in Exp. 2 was about the same as in Exp. 1.

Exp. 3. The same experiment as described above was made with $3N$ NaCl instead of K_2SO_4. In this solution after 2 days the vitreous underwent a decrease in volume to 70 % of the original. After removal of the NaCl in distilled water an increase in volume again set in; the final volume which was obtained was about 200 % (Fig. 2, curve 3). It should be noted that in concentrated NaCl solutions the decrease in volume is rather limited, and that here again the influence of the salt is completely reversible.

Exp. 4. Comparative investigations were then made with concentrated ($3N$) KCNS solutions. After measuring the volume of the fresh vitreous bodies, they were placed in the solution; a very rapid decrease was observed in the volume which after 6 hours was reduced to about 6 % of the original, after 24 hours to 1 % and after 72 hours to 0·5 %. This change of volume is shown by curve 4 of Fig. 2.

Exp. 4 a. Two vitreous bodies which after 6 hours in the KCNS solution had decreased to 6 % of their original volume were placed in distilled water which was several times renewed. After removal of the KCNS an increase in volume was observed which after 72 hours reached a maximum of only 40 % of the original (Fig. 2, curve 4*a*).

Exp. 4 b. The same experiment was repeated with some vitreous bodies left in KCNS solutions for 72 hours when the volume was 0·5 % of the original. These showed the phenomenon to a still greater extent, since after removal of the salt in distilled water, a final volume of only 4 % of the original was obtained (Fig. 2, curve 4 *b*).

Discussion.

In discussing the experiments mentioned above, attention should first be paid to the different behaviours of the vitreous towards KCNS on the one hand and towards K_2SO_4 and NaCl on the other, as is shown by the curves 4, 2 and 3 of Fig. 2. If, as Duke-Elder & Davson thought, the rapid decrease in volume in KCNS were due to simple dehydration of the fibrils of the vitreous, this noticeable difference in the curves would remain unexplained. It is evident that the observations concerning the action of KCNS point more to peptization than to simple dehydration. By such peptization the greater part of the fibrillar proteins (and perhaps also of the other proteins of the vitreous) would be dissolved in the surrounding liquid so that naturally the residue of the vitreous after washing with distilled water would regain a smaller volume than after treatment with K_2SO_4 or NaCl. Here it must be repeated that this view is in complete accordance with the observations on the action of concentrated KCNS solutions on the ultramicroscopic aspect of the structure of the vitreous as mentioned at the beginning of this paper. In this connexion it is to be noted that Duke-Elder & Davson reported increased extensibility of the structure of the vitreous after treatment with KCNS, which in my opinion can only be ascribed to increase in hydration of the fibrils. In this respect the conclusion of the authors about the dehydrating action of KCNS is not in keeping with the results of their own investigations on the elasticity of the vitreous.

SUMMARY.

A brief report is given of investigations of the action of concentrated KCNS solutions on the volume of the vitreous body, in which it is shown that the decrease in volume after treatment with KCNS is largely irreversible. This result is in accordance with earlier investigations of the influence of KCNS on the ultramicroscopic aspect of the vitreous, and with the result of experiments by Duke-Elder & Davson on the extensibility of the framework of the vitreous after treatment with this salt. The experiments indicate that in greater concentrations KCNS acts as hydrating agent and in the last resort causes peptization of the greater part of the vitreous proteins.

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CCXCII. ASCORBIC ACID OXIDASE FROM DRUMSTICK, *MORINGA PTERYGOSPERMA*.[1]

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THE high content in ascorbic acid of the pods of the drumstick tree, *Moringa pterygosperma*, was demonstrated by Damodaran & Srinivasan [1935], using the Tillmans-Harris method. The starting point of the present investigation was the observation that the press juice from this material, in contrast to its trichloroacetic acid extract, exercised no reducing action on 2:6-dibromophenolindophenol. That this anomalous behaviour was due to a vigorous enzymic oxidation of ascorbic acid in the press juice became evident from further experiments which showed, in the first place, that the juice when added to other normally stable sources of ascorbic acid such as orange juice or the press juice of *Phyllanthus emblica* caused a rapid diminution in their ascorbic acid content and secondly that if the drumstick pods in the intact condition were first immersed for a short time in boiling water, the juice obtained on pressing had a Tillmans titre practically equivalent to, or slightly higher than, that of a trichloroacetic acid extract.

Studies on the enzymic oxidation of ascorbic acid were begun by Szent-Györgyi [1928] who concluded that the oxidation of ascorbic acid by plant tissues was due to peroxidase acting through the intermediary of a phenolic compound, the phenol being first oxidized by peroxidase to the quinone which in turn oxidized the ascorbic acid, presumably without the intervention of a second enzyme. In later communications [1930; 1931] he modified this view and postulated the existence in cabbage juice of a specific "hexoxidase" which was responsible for the oxidation of ascorbic acid. From a study of the kinetics of the reaction which showed that the rate of oxidation increased with increasing concentrations of substrate, the conclusion was drawn that this oxidizing enzyme was of a complex nature and involved the intervention of an intermediate substance "*x*".

Zilva [1934] also described experiments to show that Bramley's seedling apples contained an enzyme, distinct from and unconnected with peroxidase, which was capable of reversibly oxidizing vitamin C and which he considered to be similar in nature to Szent-Györgyi's "hexoxidase".

During the course of the present work Tauber *et al.* [1935] have demonstrated the presence in the pericarp of *Cucurbita maxima* of a specific oxidase for ascorbic acid, which differs in many respects from Szent-Györgyi's "hexoxidase".

The enzyme from *M. pterygosperma* now described presents few analogies with Szent-Györgyi's "hexoxidase", but appears to be similar in most respects to that obtained by Tauber *et al.* from *C. maxima*. In the presence of molecular oxygen ascorbic acid is oxidized by it directly and specifically to its reversible first oxidation product; there is no evidence of the intervention of an intermediate carrier. A very active concentrate capable of quantitatively oxidizing large amounts of ascorbic acid in an extremely short time is readily obtained by fractional precipitation of the press juice with ammonium sulphate. Such a preparation has no action upon guaiacum, benzidine, catechol, pyrogallol or *p*-phenylenediamine, showing the absence of a polyphenol or an indophenol oxidase. On the other hand, it always shows peroxidase activity, blueing guaiacum in the presence of H_2O_2. The differential action of inhibiting agents, however, leaves no doubt of the fact that the oxidation of ascorbic acid is entirely independent of the presence of peroxidase. Thus, after treatment with acetone or alcohol the preparation has little oxidizing action on ascorbic acid, though the peroxidase remains unimpaired (Table II). On the other hand, 10^{-4} *M* cyanide completely inhibits the reaction with guaiacum and H_2O_2, while 65 % of the original capacity to oxidize ascorbic acid is still retained (Table III and Fig. 2). Further, in the press juice of the radish, *Raphanus sativus*, which contains both ascorbic acid and a peroxidase, the latter is without action on the former. Szent-Györgyi also showed [1928] that a purified peroxidase preparation is without action on ascorbic acid. In the present instance the usual colour reactions gave no evidence of the presence of a phenol or a quinone. No mention is made by Tauber *et al.* [1935] as to the presence or absence of peroxidase in their ascorbic acid oxidase preparation from *C. maxima*.

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[1] First appeared as a letter in *Current Science*, 4, 407 (1935).

A study of the course of the oxidation of ascorbic acid by the enzyme from drumstick reveals that it is unimolecular (Table IV). The rate of oxidation is proportional to enzyme concentration (Table VI) and is unaffected by increasing quantities of substrate.

In one important respect, viz., in its sensitiveness to cyanide, ascorbic acid oxidase from drumstick differs from the corresponding enzyme in *C. maxima*, as well as from "hexoxidase". While both the latter enzymes have been shown to be affected only by high concentrations of cyanide (0·01 %), the activity of the enzyme from drumstick is greatly inhibited by 10^{-3} *M* cyanide or H_2S (Table III and Fig. 2). Even 10^{-5} *M* cyanide has a definite inhibitory effect.

EXPERIMENTAL.

Preparation of the enzyme. From preliminary experiments it was found that by treating the pressed juice of drumstick with solid $(NH_4)_2SO_4$ to 1/9 saturation (5 %), an inert precipitate was thrown down, while the resulting filtrate contained almost the whole of the original enzyme activity; further saturation of the active filtrate with $(NH_4)_2SO_4$ to 3/4 saturation (35 % final concentration) precipitated most of the enzyme, the filtrate being practically inactive. The following procedure was therefore adopted in preparing the enzyme.

About 15–20 drumsticks, weighing nearly 700 g. with the outer skin scraped off, were passed through a mincer, the juice pressed out and centrifuged for 20 min. The greenish yellow supernatant liquid (about 400 ml.) was cooled in ice and $(NH_4)_2SO_4$ added to a concentration of 5 %. The heavy precipitate settling down on standing in the ice-chest overnight was filtered off and the resulting brownish yellow filtrate further treated with $(NH_4)_2SO_4$ to a final concentration of 35 %. The fine precipitate which appeared was separated by centrifuging (3000 r.p.m.) for half an hour, dispersed in about 50 ml. of water and filtered. The clear yellow filtrate thus obtained possessed powerful enzyme activity and was the material used for all experiments described here. Kept under toluene in the refrigerator, such a preparation retained its activity undiminished for over a month.

Methods.

Water, redistilled four times in all-glass (pyrex) apparatus was used for making up all the experimental solutions. A solution of ascorbic acid (B.D.H.) prepared in glass-distilled water and brought to *p*H 5·3 (*v. infra*) with Na_2CO_3 served as substrate, the exact strength of this solution being determined by titration against standard iodine (*N*/200).

Table I. *Oxidation of ascorbic acid at different* p*H values.*

McIlvaine's citric acid-phosphate buffer		Sørensen's phosphate buffer	
*p*H	Ascorbic acid oxidized %	*p*H	Ascorbic acid oxidized %
3·0	27·94	5·3	100·00
3·4	55·89	5·6	100·00
3·8	76·47	5·9	96·16
4·2	85·31	6·5	84·63
4·6–5·6	89·71	6·8	67·32
5·8	82·75	7·2	42·32
6·0	81·04	8·0	23·08
6·4	68·96	—	—
6·8	44·82	—	—
7·2	34·48	—	—
7·6	24·14	—	—

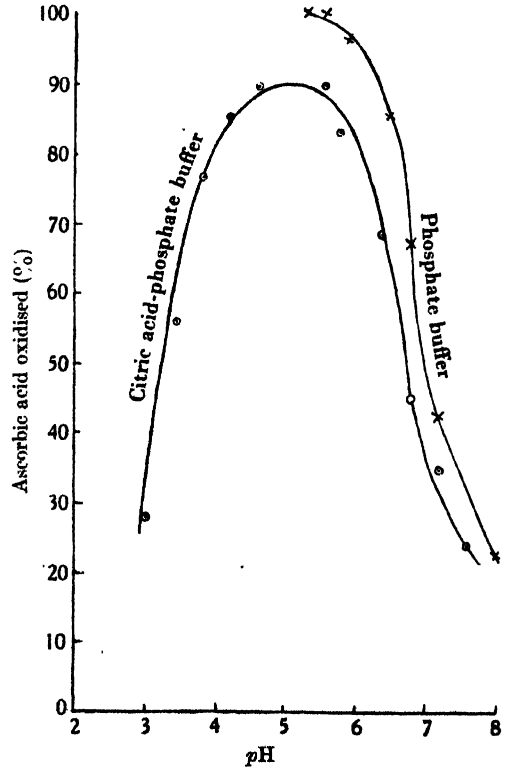

Fig. 1. Activity-*p*H curves for ascorbic acid oxidase.

The reaction mixture was contained in 50 ml. beakers held in a thermostat at 37°. After the addition of enzyme and during its action, the contents of the beaker were kept well stirred. Enzyme action was arrested, when desired, by adding acetic acid to the reaction mixture, in which unoxidized ascorbic acid was determined at any time by titrating against a standard solution of 2:6-dibromophenolindophenol.

Control experiments were run under identical conditions, but with the boiled enzyme. Though stock solutions of ascorbic acid prepared as described suffered no oxidation on standing for two or three days in stoppered bottles, the control experiments, conducted as they were in open beakers and with the probable metallic contamination from the boiled enzyme showed small decreases in the Tillmans titres. Such decreases were extremely minute compared with those caused by the enzyme; the corresponding corrections were, however, made in the experimental values.

The optimum pH *for enzyme action.*

McIlvaine's phosphate-citric acid and Sørensen's phosphate buffers were used for obtaining solutions of various reactions. The oxidation taking place in 3 min. at 37° in 5 ml. of a reaction mixture composed of 0·4 mg. ascorbic acid, 0·1 ml. enzyme and 1 ml. of buffer solution of the desired *p*H was determined by titration of the unoxidized ascorbic acid.

From Table I and Fig. 1 it will be seen that the optimum reaction for this enzyme lies over a range *p*H 4·6–5·6 in citric acid-phosphate buffer and 5·3–5·6 in phosphate buffer, the natural *p*H of the press juice of drumstick being 5·6–5·8. Further, the extent of oxidation of ascorbic acid by the enzyme is greater in the latter than in the former buffer. In all subsequent experiments phosphate buffer of *p*H 5·3 (9·75 ml. $M/15$ NaH_2PO_4+0·25 ml. $M/15$ Na_2HPO_4 mixture according to Sørensen) was therefore used.

Nature and extent of oxidation of ascorbic acid by the enzyme.

The reaction does not proceed in the absence of oxygen, no oxidation taking place in evacuated Thunberg tubes.

The enzyme oxidizes ascorbic acid to its reversible oxidation product as is evident from the following experiment: 0·5398 mg. ascorbic acid was completely oxidized (as judged by a zero Tillmans titre in a duplicate experiment) by 0·1 ml. enzyme at *p*H 5·3 at 37°. Through the oxidized solution H_2S was bubbled for 10 min. and the solution kept overnight in a stoppered flask. H_2S was then completely removed by a current of carbon dioxide; the solution then had an ascorbic acid content of 0·5202 mg.

Action of the enzyme on other compounds.

Guaiacum, benzidine, catechol, pyrogallol and *p*-phenylenediamine were not affected by the enzyme preparation, suggesting that the enzyme, which oxidizes ascorbic acid, is not a polyphenol or an indophenol oxidase.

All these substances were oxidized by the enzyme preparation in the presence of H_2O_2, indicating the presence of peroxidase, but the following experiments show that the peroxidase action was totally unconnected with the oxidation of ascorbic acid.

Effect of alcohol or acetone on ascorbic acid oxidase and peroxidase.

2 ml. enzyme solution were treated in a 15 ml. centrifuge-tube with 4 vol. of 95 % ethyl alcohol, methyl alcohol or acetone. The precipitate formed in each

case was separated by centrifuging and redispersed in 2 ml. water. The supernatant liquid was evaporated at a low temperature *in vacuo* and the resulting residue also taken up in 2 ml. water. The enzymic activities of these two fractions were compared with that of the untreated enzyme preparation. The results are given in Table II.

Table II. *Effect of alcohol or acetone on ascorbic acid oxidase.*

Reaction mixture: 0·5 mg. ascorbic acid, 0·1 ml. enzyme in a total volume of 5 ml. at *p*H 5·3 at 37°.

	Ascorbic acid oxidized in 2 min. %
1. Untreated enzyme	77·52
2. Enzyme treated with ethyl alcohol	8·90
3. Enzyme treated with methyl alcohol	2·60
4. Enzyme treated with acetone	26·97
5. Filtrates from 2, 3 or 4	0·0

The enzyme is thus seen to be highly sensitive to ethyl or methyl alcohol and to a less extent to acetone. The peroxidase activity of the preparation as studied qualitatively by the colour developed with a solution of pyrogallol in the presence of H_2O_2, remained unimpaired by such treatment.

Effect of cyanide or H_2S on ascorbic acid oxidase and peroxidase.

The action of the enzyme on ascorbic acid under standard conditions was studied with and without the addition of cyanide or H_2S. Simultaneously, peroxidase activity was also studied qualitatively as before.

Table III. *Effect of cyanide or H_2S on ascorbic acid oxidase and peroxidase.*

Reaction mixture: 0·3 mg. ascorbic acid, 0·1 ml. enzyme in a total volume of 5 ml. at *p*H 5·3 at 37°.

	Ascorbic acid oxidized (%)					
Time in min.	Enzyme	Enzyme $+10^{-5}\,M$ KCN	Enzyme $+5\times10^{-5}\,M$ KCN	Enzyme $+10^{-4}\,M$ KCN	Enzyme $+10^{-3}\,M$ KCN	Enzyme $+10^{-3}\,M$ H_2S
1	33·88	29·03	25·81	22·58	6·45	6·34
2	56·45	53·23	46·77	43·55	9·68	11·11
3	75·82	70·98	67·75	64·52	12·9	14·29
4	90·33	88·72	85·49	82·26	14·52	—
Peroxidase activity	+	±	–	–	–	

From the above table and the graphs in Fig. 2, the inhibitory influence of cyanide or H_2S at low concentration is evident. Slight though definite inhibition of the oxidation of ascorbic acid takes place even at a cyanide concentration of $10^{-5}\,M$. But the effect is marked only above a concentration of $10^{-4}\,M$. Peroxidase activity, on the other hand, ceases completely long before such a concentration of cyanide is reached.

Enzyme kinetics.

Order of the reaction. The oxidation of ascorbic acid by the enzyme is a reaction of the first order, as is seen from the calculated values for the velocity constant given in the last column of Table IV. For this particular experiment a very dilute solution of 2:6-dibromophenolindophenol, equivalent to 0·05 mg. ascorbic acid per ml. was used to ensure greater accuracy.

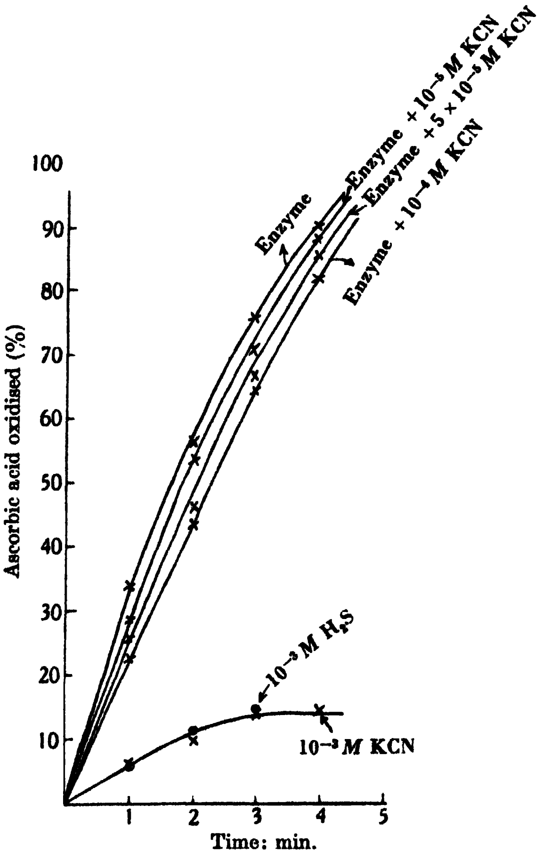

Fig. 2. Effect of KCN or H_2S on ascorbic acid oxidase.

Table IV. *Rate of oxidation of ascorbic acid by the enzyme.*

Reaction mixture: 0·41 mg. ascorbic acid, 0·1 ml. enzyme in a total volume of 5 ml. at *p*H 5·3 at 37°.

Time in min.	Ascorbic acid oxidized %	$k=\frac{1}{t}\log_{10}\frac{a}{a-x}$
1	22·67	0·11160
2	38·68	0·10650
3	52·00	0·10633
4	62·67	0·10697
5	70·68	0·10652
7	82·00	0·10639

Relation between concentration of enzyme and its action. Mixtures of 1 ml. ascorbic acid solution containing 0·41 mg. of acid, 1 ml. phosphate buffer *p*H 5·3 and varying quantities of glass-distilled water to make the final volume after the addition of enzyme 5 ml., were incubated with different quantities of enzyme at 37° and the rate of oxidation determined by titration. The results are given in Table V.

Table V. *Influence of the concentration of enzyme on the rate of oxidation of ascorbic acid.*

Time in min.	mg. ascorbic acid oxidized by			
	0·3 ml. enzyme	0·2 ml. enzyme	0·1 ml. enzyme	0·05 ml. enzyme
1	0·2033	0·1621	0·09344	0·04397
2	0·3243	0·2583	0·1597	0·08245
3	—	0·3243	0·2061	0·1099
4	—	—	0·2583	—
5	—	—	0·2913	0·1759
7	—	—	0·3380	0·2199
10	—	—	—	0·2859

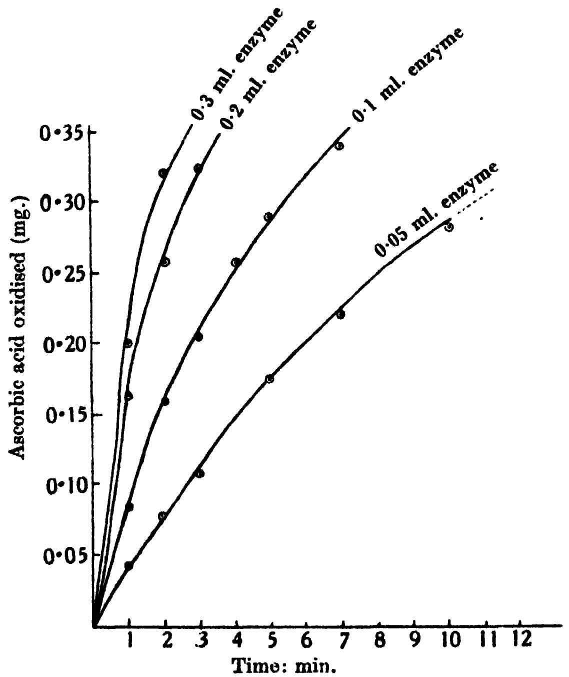

Fig. 3. Enzyme concentration and ascorbic acid oxidation.

The above results are plotted graphically in Fig. 3. From these graphs the results given in Table VI were obtained.

From Table VI, it is clear that the rate of oxidation of ascorbic acid is directly proportional to enzyme concentration.

Substrate concentration and enzyme action. 5 ml. reaction mixture composed of 1 ml. phosphate buffer *p*H 5·3, 0·2 ml. enzyme and different quantities of ascorbic acid ranging from 0·5633 to 4·893 mg. were incubated for 3 min. at 37°. The amount of ascorbic acid oxidized at the end of the interval was determined in each case. 0·4074 mg. ascorbic acid was uniformly oxidized, showing that under these conditions the rate of oxidation is unaffected by increasing quantities of substrate.

Table VI. *Enzyme concentration and ascorbic acid oxidation.*

Enzyme quantity ml. (E)	Time in min. (T)	E × T	Ascorbic acid oxidized mg.
0·30	1·0	0·3	0·2150
0·30	2·0	0·6	0·3125
0·20	1·5	0·3	0·2175
0·20	3·0	0·6	0·3200
0·10	3·0	0·3	0·2150
0·10	6·0	0·6	0·3150
0·05	6·0	0·3	0·2025
0·05	12·0	0·6	0·3200*

* Obtained by extrapolation.

SUMMARY.

1. From the press juice of the drumstick, *Moringa pterygosperma* an enzyme preparation has been obtained which, in presence of oxygen, oxidizes ascorbic acid to its reversible oxidation product.

2. The enzyme does not oxidize guaiacum, benzidine, catechol, pyrogallol or *p*-phenylenediamine.

3. There is present in the preparation a peroxidase which, however, has been shown to have no role in the oxidation of ascorbic acid.

4. The oxidation of ascorbic acid by the enzyme is a reaction of the first order. The rate of oxidation is directly proportional to enzyme concentration and above a certain limit is unaffected by increasing quantities of substrate.

5. From the above findings, the ascorbic acid-oxidizing action of the preparation is to be attributed to an oxidase therein specific for ascorbic acid.

6. Low concentrations of cyanide or H_2S inhibit the activity of the enzymes. It is destroyed almost completely by alcohol and partially by acetone.

The author is very grateful to Prof. M. Damodaran for his interest in the investigation.

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CCXCIII. DISSOCIATION CONSTANTS AND STRUCTURES OF GLUTAMIC ACID AND ITS ESTERS.

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It has been shown [Neuberger, 1936] that the dissociation constants of simple zwitterions which contain only one carboxyl and one amino-group are quantitatively related to their structures; similarly the change of their apparent dissociation constants with the ionic strength of the solution of the different amino-acids can be satisfactorily explained on such a basis. This paper represents an extension of the previous work to a more complex zwitterion, namely glutamic acid. It was deemed desirable to determine also the dissociation constants of the different esters of glutamic acid and in this connexion the preparation of α-ethyl hydrogen glutamate is described.

EXPERIMENTAL.

Preparation of compounds.

α-Ethyl hydrogen glutamate.[1] *N*-Benzylcarbonylglutamic anhydride [cf. Harington & Mead, 1935] (20 g.) and alcohol (100 ml.) were heated in a sealed tube for 3½ hours at 125°; the solution was concentrated to low bulk, diluted with ether and extracted with aqueous sodium bicarbonate. The aqueous solution was acidified and extracted several times with ether; the combined ethereal extracts were dried and evaporated leaving an oil which slowly crystallized. After recrystallization from ether the *α-ethyl hydrogen* N-*benzylcarbonylglutamate* thus formed had M.P. 100°. Yield 25%. (Found: N, 4·58%. $C_{15}H_{19}O_6N$ requires: N, 4·53%. 22·1 mg. required 3·6 ml. *N*/50 NaOH for neutralization to cresol red; $C_{15}H_{19}O_6N$ titrating as a monobasic acid requires 3·57 ml.)

The above α-ethyl hydrogen *N*-benzylcarbonylglutamate (4·5 g.), dissolved in a little 80% alcohol containing HCl (1 equiv.) was reduced with hydrogen and palladium black; the filtered solution was evaporated *in vacuo* and the residue taken up in a little 90% alcohol, adjusted to *p*H 6·0 with lithium hydroxide and placed in a desiccator at 400 mm. pressure over a dish of absolute alcohol. Crystallization soon set in and was complete in 2 days. Recrystallization in a similar manner yielded *α-ethyl hydrogen glutamate* having M.P. 110°. (Found: N, 7·85%. $C_7H_{13}O_4N$ requires N, 8·01%.) The ester is very soluble in water, aqueous alcohol and aqueous acetone, but only sparingly soluble in absolute alcohol.

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[1] Treatment of *N*-benzylcarbonylglutamic anhydride with sodium ethoxide in the cold according to Melville [1934] gives a mixture of α- and γ-esters; thus a sample of the oily product so obtained yielded, on hydrogenation, approximately 30% of γ-ethyl hydrogen glutamate, whilst another sample on long keeping, deposited crystals of α-ethyl hydrogen *N*-benzylcarbonylglutamate; the different treatment described above, which gives a more uniform product, was therefore adopted.

γ-Ethyl hydrogen glutamate was prepared according to Bergmann & Zervas [1933].

Ethyl glutamate hydrochloride was obtained by esterification of glutamic acid in the usual manner and was twice recrystallized.

Glutamic acid was a carefully recrystallized commercial specimen.

Potentiometric methods.

Measurements were made as previously described [Neuberger, 1936]. The liquid junctions which were made inside a cylindrical tube of 3 mm. internal diameter were initially very sharp; the potentials were generally constant for many hours within 0·1 mv. The esters, which were very stable at acid reaction, were hydrolysed with varying ease in alkaline media; in order to prevent local excess of alkali therefore NaOH was added to the mechanically stirred solution of the ester and the mixture transferred to the cell, mixing and transference being effected in an atmosphere of hydrogen freed from traces of CO_2. Readings were taken after suitable intervals; the potentials were plotted against time and extrapolation to zero time was made. The initial drift in potential was about 1 mv. per 10 min. in the case of pK_2 of γ-ethyl hydrogen glutamate at a pH of about 8·8 and 1–2 mv. per hour in the case of ethyl glutamate at a pH of about 7·2; α-ethyl hydrogen glutamate was quite stable under the conditions of the measurements.

Results.

In Table I the results of the potentiometric measurements are recorded. Column 1 gives the potentials corrected for barometric pressure; in column 2 the potentials are recorded after correction for liquid junction. In column 3, a denotes the degree of neutralization, c in column 4 gives the total concentration of the acid or base present. The ionic strength was varied by addition of KCl. The apparent dissociation constants were determined as described; the values are generally accurate within ±0·01. In the case of a very low pK (pK_1 of glutamic acid and of γ-ethyl hydrogen glutamate) the large blank correction causes the margin of error to be higher. In the cases of pK_2 of γ-ethyl hydrogen glutamate and pK of ethyl glutamate measurements are also less reliable owing to hydrolysis and/or lactam formation. The value of $pK_3{}^0$ of glutamic acid is also only accurate ±0·03, since the correction for ionic strength is larger.

DISCUSSION.

The dissociation constants of glutamic acid.

Several equilibria are involved in the dissociation of glutamic acid and it is necessary to consider these in some detail. Glutamic acid can be regarded as a tribasic acid of which the most acidic form is a cation. The first step consists in the dissociation of H^+ from the cation according to one of the three reactions:[1]

$$\underset{a}{\mathrm{HOOC.CH(NH_3^+)CH_2.CH_2.COOH}} \underset{\rightleftharpoons}{\rightleftharpoons} \underset{b_1}{\mathrm{{}^-OOC.CH(NH_3^+)CH_2.CH_2.COOH}} + \underset{h}{\mathrm{H^+}} \quad \text{......(1}a\text{)},$$

$$\underset{a}{\mathrm{HOOC.CH(NH_3^+)CH_2.CH_2.COOH}} \underset{\rightleftharpoons}{\rightleftharpoons} \underset{b_2}{\mathrm{HOOC.CH(NH_3^+)CH_2.CH_2.COO^-}} + \underset{h}{\mathrm{H^+}} \quad \text{......(1}b\text{)},$$

$$\underset{a}{\mathrm{HOOC.CH(NH_3^+)CH_2.CH_2.COOH}} \underset{\rightleftharpoons}{\rightleftharpoons} \underset{b_3}{\mathrm{HOOC.CH(NH_2)CH_2.CH_2.COOH}} + \underset{h}{\mathrm{H^+}} \quad \text{......(1}c\text{)}.$$

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[1] The letters *a*, *b* etc. beneath the equations are arbitrary signs to denote the different ionic species and are used for brevity in the equations developed later in this paper.

Table I. *Potentiometric measurements.*

	E	$E_{corr.}$	a	c	μ	pH	pK
Glutamic acid.							
pK_1	495·3	493·4	50·0	0·008	0·008	2·683	2·129
	495·8	494·4	50·0	0·008	0·020	2·705	2·146
	497·7	497·5	50·0	0·008	0·084	2·757	2·188
$pK_1^0 = 2·155$ (corrected).							
pK_2	590·9	589·5	51·0	0·008	0·008	4·313	4·293
	564·5	563·1	25·0	0·008	0·006	3·867	4·301
	590·7	589·4	51·0	0·008	0·014	4·311	4·288
	589·4	588·3	51·0	0·008	0·020	4·292	4·262
	588·0	587·3	51·0	0·008	0·044	4·275	4·244
	585·0	584·7	51·0	0·008	0·084	4·232	4·198
	618·4	617·0	75·0	0·008	0·008	4·772	4·295
$pK_2^0 = 4·324$ (corrected).							
pK_3	920·4	919·0	55·5	0·008	0·016	9·883	9·807
	893·7	892·3	29·5	0·008	0·012	9·432	9·818
	947·9	946·5	79·8	0·008	0·021	10·348	9·813
	909·1	909·4	55·5	0·008	0·173	9·721	9·641
	912·0	911·8	55·5	0·008	0·096	9·762	9·683
	913·9	913·3	55·5	0·008	0·060	9·787	9·716
	915·6	914·7	55·5	0·008	0·040	9·813	9·741
$pK_3^0 = 9·960$.							
α-Ethyl hydrogen glutamate.							
pK_1	562·4	560·9	55·2	0·00725	0·004	3·829	3·881
	562·9	561·4	55·2	0·00725	0·009	3·837	3·888
	563·2	562·3	55·2	0·00725	0·014	3·853	3·905
	563·6	562·9	55·2	0·00725	0·02	3·863	3·915
	565·6	565·2	54·6	0·00725	0·044	3·902	3·946
	563·6	563·4	55·9	0·00725	0·084	3·870	3·931
	590·7	589·3	27·6	0·00725	0·002	4·311	3·876
	534·5	533·1	80·3	0·00680	0·006	3·359	3·871
	535·5	534·1	80·3	0·00725	0·008	3·376	3·886
$pK_1^0 = 3·846$.							
pK_2	770·8	769·4	73·5	0·00725	0·0058	7·355	7·801
	769·7	768·6	73·5	0·00725	0·02	7·340	7·786
	768·2	767·6	73·5	0·00725	0·042	7·323	7·766
	744·0	742·6	88·8	0·00725	0·005	6·901	7·800
	770·5	769·4	73·7	0·008	0·008	7·354	7·802
	771·4	770·4	73·1	0·008	0·014	7·372	7·806
	769·0	768·7	72·5	0·00725	0·048	7·341	7·760
$pK_2^0 = 7·838$.							
γ-Ethyl hydrogen glutamate.							
pK_1	503·4	501·4	31·8	0·01257	0·008	2·823	2·184
	523·3	521·4	15·9	0·01257	0·008	3·151	2·185
	490·4	488·4	47·7	0·01257	0·008	2·605	2·172
	502·4	500·9	31·8	0·01257	0·014	2·817	2·164
	496·4	495·6	50·0	0·008	0·025	2·725	2·180
	497·3	496·8	50·0	0·008	0·044	2·745	2·200
	499·1	498·2	50·0	0·008	0·084	2·769	2·223
	499·3	499·6	50·0	0·008	0·244	2·793	2·207
$pK_1^0 = 2·148$.							
pK_2	846·6	845·1	76·0	0·008	0·008	8·635	9·141
	874·4	873·0	52·0	0·008	0·008	9·104	9·168
	859·5	858·1	66·4	0·008	0·008	8·854	9·169
	840·0	838·6	80·8	0·008	0·008	8·524	9·150
$pK_2^0 = 9·19$.							
Ethyl glutamate.							
	752·4	751·0	52·1	0·008	0·008	7·093	7·080
	753·0	751·9	52·1	0·008	0·016	7·053	7·095
	753·7	752·9	52·1	0·008	0·024	7·075	7·112
	756·8	756·5	52·1	0·008	0·084	7·136	7·172
	722·0	720·6	76·1	0·008	0·008	6·530	7·065
$pK^0 = 7·035$.							

Reactions (1*a*) and (1*b*) lead to zwitterions, whilst reaction (1*c*) produces an uncharged molecule. For electrostatic reasons most of the cations will react according to (1*a*) rather than (1*b*). From what is known of the order of magnitude of the dissociation of the amino-group in amino-acids and their esters and especially from the relative values of the dissociation constants of the esters of glutamic acid given in this paper it can be assumed that the dissociation of the NH_3^+ group is negligible compared with the dissociation of either of the two carboxyl groups. Reaction (1*c*) can therefore be neglected and the second step can be written as follows:

$$\underset{b_1}{{}^-OOC.CH(NH_3^+)CH_2.CH_2.COOH} \rightleftarrows \underset{c}{{}^-OOC.CH(NH_3^+)CH_2.CH_2.COO^-} + \underset{h}{H^+} \quad ......(2a),$$

$$\underset{b_2}{HOOC.CH(NH_3^+)CH_2.CH_2.COO^-} \rightleftarrows \underset{c}{{}^-OOC.CH(NH_3^+)CH_2.CH_2.COO^-} + \underset{h}{H^+} \quad ......(2b).$$

In order to obtain the individual equilibrium constants of the reactions (1*a*), (1*b*), (2*a*) and (2*b*) certain additional assumptions must be made, since actual measurements only give overall constants K_1 and K_2 which may be defined by the equations

$$K_1 = \frac{(b_1+b_2)\,h}{a} \quad ......(3), \qquad K_2 = \frac{c.h}{(b_1+b_2)} \quad ......(4).$$

In glutamic acid K_1 is only about 100 times larger than K_2; the large value of K_1 renders it necessary to perform measurements for the determination of K_1 at *p*H values where reactions (2*a*) and (2*b*) cannot be neglected. The formula generally used for the calculation of dissociation constants from potentiometric measurements therefore leads to incorrect results.

In order to correct for the effect of overlapping of the two steps of dissociation we proceed as follows. To a solution of ionic strength μ containing A mol. of glutamic acid we add B mol. of HCl or NaOH respectively, B having a positive sign if acid is added and a negative sign if alkali is added. Since the solution as a whole is electrically neutral we have then, neglecting OH^-,

$$a - c + h = B \quad ......(5),$$

and further

$$-\log h = pH - \frac{0{\cdot}5\sqrt{\mu}}{1+\sqrt{\mu}} \quad ......(6),$$

$$a + b_1 + b_2 + c = A \quad ......(7).$$

By combining (3), (4), (5) and (7) we get

$$A = \frac{(B-h)\,(h^2 + K_1 h + K_1 K_2)}{h^2 - K_1 K_2}.$$

If we now write for convenience

$$\frac{A}{B-h} = m,$$

we get

$$K_1 = \frac{(m-1)\,h^2}{(m+1)\,K_2 + h} \quad ......(8).$$

In order to determine K_1 and K_2 at least two measurements with differing m and h must be made. If solution I contains A mol. of glutamic acid, B_1 mol. of HCl ($B_1 < A$) and h_1 mol. of H^+ while solution II contains A mol. of glutamic acid, $-B_2$ mol. of NaOH (absolute value of $B_2 < A$) and h_2 mol. of H^+, we then have

$$K_1 = \frac{(m_1-1)\,h_1^2}{(m_1+1)\,K_2 + h_1} = \frac{(m_2-1)\,h_2^2}{(m_2+1)\,K_2 + h_2}.$$

From this expression we get finally

$$K_2 = h_1 h_2 \frac{(m_2 - 1)\,h_2 - (m_1 - 1)\,h_1}{(m_2 + 1)(m_1 - 1)\,h_1^2 - (m_1 + 1)(m_2 - 1)\,h_2^2} \quad \text{......(9),}$$

and similarly

$$K_1 = \frac{(m_2 + 1)(m_1 - 1)\,h_1^2 - (m_1 + 1)(m_2 - 1)\,h_2^2}{(m_2 + 1)\,h_1 - (m_1 + 1)\,h_2} \quad \text{......(9}a\text{).}$$

It can be seen that if h_2 is small compared with h_1 equations (9) and (9*a*) give the formulae for an ordinary acid. The values obtained from (9) and (9*a*) have to be corrected for activity in order to give the thermodynamic constants. For small ionic strengths it can be assumed that the activity coefficient of the zwitterionic form of glutamic acid can be neglected and that the activity coefficients of H^+ and of the cation of glutamic acid are equal. pK_1^0 is then equal to the negative logarithm of the "concentration constant". pK_2^0 is given by (10):

$$pK_2^{\circ} = -\log K_2 + \frac{\sqrt{\mu}}{1 + \sqrt{\mu}} \quad \text{......(10).}$$

The correction introduced by employing equations (9) and (9*a*) instead of the formulae for an ordinary acid is under the conditions of the experiments about 0·08 for pK_1^0 and only 0·01 for pK_2^0.

As pointed out above the constants pertaining to the individual reactions (1*a*), (1*b*), (2*a*) and (2*b*) cannot be obtained from the actual measurements; but the constants of the individual reactions K_1', K_1'', K_2' and K_2'' are related to the overall constants K_1 and K_2 by the equations:

$$K_1 = K_1' + K_1'' \quad \text{......(11),}$$

$$\frac{1}{K_2} = \frac{1}{K_2'} + \frac{1}{K''_2} \quad \text{......(11}a\text{),}$$

[*vide* Wegscheider, 1895; Adams, 1916; Bjerrum, 1923].

From (3), (4), (11) and (11*a*) we get

$$\frac{K_1'}{K_1''} = \frac{K_2''}{K_2'} = \frac{b_1}{b_2} = q \quad \text{......(12).}$$

It is well known that the effect of COOH as substituent on the dissociation of another carboxyl group is more or less equal to the effect of COOEt. We may assume therefore that K_1'' is equal to the first dissociation constant of α-ethyl hydrogen glutamate. If this assumption is correct the value obtained for K_1' should be equal to K_1 of γ-ethyl hydrogen glutamate. Actually, we find $pK_1' = 2{\cdot}164$, whilst the experimental value for pK_1^0 of γ-ethyl hydrogen glutamate is 2·143. From (11*a*) we get $pK_2'' = 4{\cdot}315$ and $pK_2' = 2{\cdot}633$.

It has been pointed out by Wegscheider [1895] that for a symmetrical dibasic acid K_1, the overall constant must be for statistical reasons twice as large as K_1', the individual constant of each group. For an unsymmetrical dibasic acid, however, the statistical factor is very small [Bjerrum, 1923]. This is shown very well in the case of glutamic acid, where one carboxyl group is adjacent to NH_3^+ and the second carboxyl removed therefrom by two carbon atoms. Here the statistical factor causes an increase only of about 2 % in K_1.

A solution of glutamic acid contains, apart from several ionic species, two types of zwitterions resembling respectively α- and γ-aminobutyric acid. Their ratio is equal to q which is about 48, *i.e.* most of the zwitterions are of the α-amino-acid type.

Change of apparent dissociation constant with ionic strength.

The first dissociation constant of glutamic acid should, at low ionic strength, show the behaviour of a cationic acid, since the activity coefficient of the zwitterionic form can be neglected under these conditions; the second constant on the other hand, should be that of an ordinary acid. Experimental data show that this is approximately the case. The third step in the dissociation is represented by the formula

$$^{-}OOC.CH(NH_3^+)CH_2.CH_2.COO^- \rightleftharpoons {}^{-}OOC.CH(NH_2).CH_2.CH_2.COO^- + H^+.$$

It may be assumed that in the first ion the two opposite charges, NH_3^+ and α-COO^-, neutralize each other and the ion can be regarded as univalent, whilst the second ion is bivalent. We therefore should have

$$pK_3^0 = pK_3 + \frac{1\cdot5\sqrt{\mu}}{1+\sqrt{\mu}}.$$

But the experimental data do not fit this formula satisfactorily. This may be due to the fact that the ionic diameter of the bivalent ion is rather large and the simple formula is only approximately correct in the case where the two charges of the bivalent ion are not far apart. If the ionic diameter is assumed to be 6 Å., which is a very probable value, we get excellent agreement as shown in

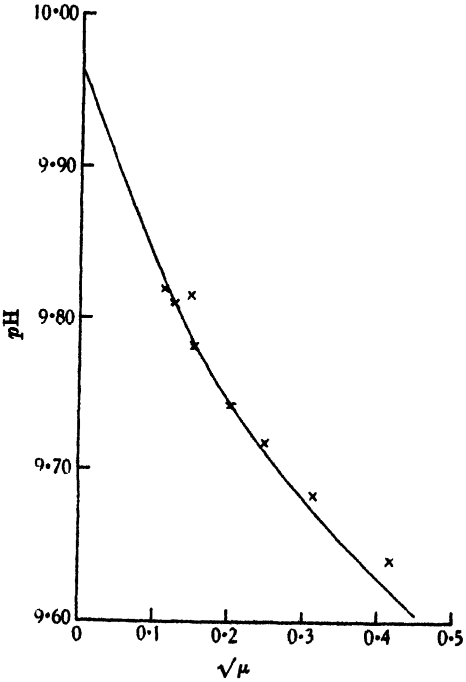


Fig. 1. Variation of pK_3 of glutamic acid with ionic strength of solution. The curve is drawn according to the equation $pK_3 = 9\cdot96 - \frac{1\cdot5\sqrt{\mu}}{1+2\sqrt{\mu}}$; the crosses represent experimental observations.

Fig. 1. The formula used for the extrapolation of pK_3^0 can now be written in the form

$$pK_3^0 = pK_3 + \frac{1\cdot5\sqrt{\mu}}{1+2\sqrt{\mu}}.$$

In all other cases the simple formulae for cationic acids and ordinary acids can be applied, since the activity coefficients of the zwitterions can be neglected with the small ionic strengths used.

Dissociation constants and structures.

Table II gives the dissociation constants of glutamic acid, its esters and of a number of related compounds. The dissociation constant of ethyl hydrogen glutarate has not been measured, but it can be estimated by using the rule discovered by Wegscheider [1895] that K of a half-ester of a symmetrical dibasic acid is half as large as the first constant of the acid. Such an estimate which can be regarded as correct within ±0·04 leads to pK_{ester} of 4·638, and fits quite well into the series of dissociation constants of the half-esters of the paraffin dicarboxylic acids, ethyl hydrogen succinate having pK 4·521 and ethyl hydrogen suberate pK 4·836. For the first pK of glutamic acid the value used in Table II is that of pK_1' and for the second pK that of pK_2''.

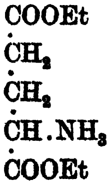
Table II. *Dissociation constants.*

	First COOH	Second COOH	NH_3
n-Butyric acid	4·820	—	—
Glutaric acid	4·337*	5·420*	—
Ethyl hydrogen glutarate	4·638†	—	—
Alanine	2·340	—	9·870
γ-Amino-*n*-butyric acid	4·230	—	10·430
Ethyl γ-amino-*n*-butyrate	—	—	9·710
Glutamic acid	2·164†	4·315†	9·960
α-Ethyl hydrogen glutamate	3·847	—	7·838
*iso*Glutamine	3·81‡	—	7·88‡
γ-Ethyl hydrogen glutamate	2·148	—	9·19
Glutamine	2·17‡	—	9·13‡
Ethyl glutamate	—	—	7·035
Glycine ester	—	—	7·75

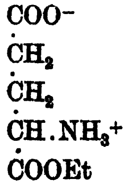
* Gane and Ingold [1931]. † See text. ‡ Melville & Richardson [1935].
For other data *vide* Neuberger [1936] and this paper.

These dissociation constants furnish a very good example of the quantitative relationship existing between ionization and structure. The first pK of glutamic acid and pK_1 of γ-ethyl hydrogen glutamate are practically equal which is to be expected if we assume that the effect of COOEt is equal to that of COOH. Both pK values are decreased by 0·176 or 0·191 respectively as compared with pK_1 of an α-amino-acid such as alanine. This effect is quantitatively the same as that of the second COOH in glutaric acid on pK_1', this alteration in magnitude being $pK_{\text{butyric acid}} - pK_{1\,\text{glutaric acid}} - \log 2 = 0{\cdot}182$. The influence of the carboxyl group on NH_3^+ is larger; the introduction of a carboxyl in the γ-position into an α-amino-acid ester as in α-ethyl hydrogen glutamate decreases the pK of NH_3^+ by 0·735 (difference between $pK_{\text{glycine ester}}$ and $pK_{\text{ethyl glutamate}}$) which is similar to the influence of introduction of COOEt into the γ-position of butylamine as in ethyl γ-aminobutyrate. Another interesting point is that pK values of α-ethyl hydrogen glutamate and *iso*glutamine on the one hand and of γ-ethyl hydrogen glutamate and glutamine on the other are nearly identical.

The influence of the charges on the pK values is even more interesting. The difference between pK_2 of α-ethyl hydrogen glutamate and pK of ethyl glutamate is 0·803 and is due to the influence of the negative charge of the carboxyl group in α-ethyl hydrogen glutamate.

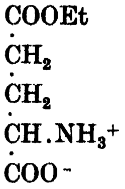


$$\begin{array}{l} \mathrm{COOEt} \\ \cdot \\ \mathrm{CH_2} \\ \cdot \\ \mathrm{CH_2} \\ \cdot \\ \mathrm{CH.NH_3^+} \\ \cdot \\ \mathrm{COOEt} \end{array}$$

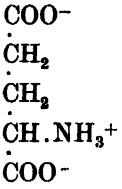


$$\begin{array}{l} \mathrm{COO^-} \\ \cdot \\ \mathrm{CH_2} \\ \cdot \\ \mathrm{CH_2} \\ \cdot \\ \mathrm{CH.NH_3^+} \\ \cdot \\ \mathrm{COOEt} \end{array}$$

This difference should be equal to that between pK_3 of glutamic acid and pK_2 of γ-ethyl hydrogen glutamate; the figure actually found is 0·75.

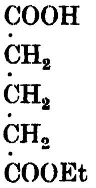


$$\begin{array}{l}\mathrm{COOEt}\\ \mathrm{CH_2}\\ \mathrm{CH_2}\\ \mathrm{CH.NH_3^+}\\ \mathrm{COO^-}\end{array}$$

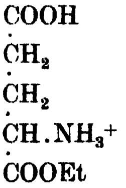


$$\begin{array}{l}\mathrm{COO^-}\\ \mathrm{CH_2}\\ \mathrm{CH_2}\\ \mathrm{CH.NH_3^+}\\ \mathrm{COO^-}\end{array}$$

The influence of the negatively charged γ-carboxyl group on NH_3^+ should be opposite in sign but equal in absolute value to the influence of NH_3^+ on the γ-carboxyl group. The difference between pK_1 of α-ethyl hydrogen glutamate and ethyl hydrogen glutarate is 0·791, which is in very satisfactory agreement.

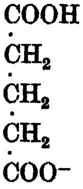


$$\begin{array}{l}\mathrm{COOH}\\ \mathrm{CH_2}\\ \mathrm{CH_2}\\ \mathrm{CH_2}\\ \mathrm{COOEt}\end{array}$$

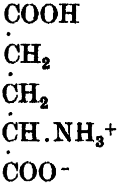


$$\begin{array}{l}\mathrm{COOH}\\ \mathrm{CH_2}\\ \mathrm{CH_2}\\ \mathrm{CH.NH_3^+}\\ \mathrm{COOEt}\end{array}$$

Comparison between pK_2' of glutaric acid ($pK_2 - \log 2$) and pK_2 of glutamic acid leads to the similar value of 0·804.

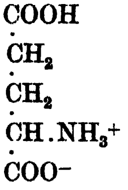


$$\begin{array}{l}\mathrm{COOH}\\ \mathrm{CH_2}\\ \mathrm{CH_2}\\ \mathrm{CH_2}\\ \mathrm{COO^-}\end{array}$$

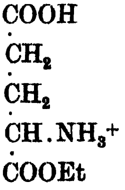


$$\begin{array}{l}\mathrm{COOH}\\ \mathrm{CH_2}\\ \mathrm{CH_2}\\ \mathrm{CH.NH_3^+}\\ \mathrm{COO^-}\end{array}$$

The influence of the negatively charged α-carboxyl on the γ-carboxyl group can be estimated by comparing pK_2 of glutamic acid and pK_1 of α-ethyl hydrogen glutamate.



$$\begin{array}{l}\mathrm{COOH}\\ \mathrm{CH_2}\\ \mathrm{CH_2}\\ \mathrm{CH.NH_3^+}\\ \mathrm{COO^-}\end{array}$$



$$\begin{array}{l}\mathrm{COOH}\\ \mathrm{CH_2}\\ \mathrm{CH_2}\\ \mathrm{CH.NH_3^+}\\ \mathrm{COOEt}\end{array}$$

The difference in the pK values is 0·468; the difference between pK_1 and pK_2 of glutaric acid is 0·487 after correction for statistical effect. The distances between the different groups in glutamic acid can be calculated by using Bjerrum's formula [*vide* Neuberger, 1936]. The distance between the centres of the two carboxyl groups according to this calculation is 6·5 Å. and the distance between the NH_3^+ group and the γ-carboxyl group about 4 Å. Applying the formula of Ingold [1931] the distances are 7·2 Å. and 5·2 Å. respectively. These values can only be regarded as approximate, but it can safely be concluded that the distance between NH_3^+ and the γ-carboxyl group is considerably smaller than the distance between the two carboxyl groups. Such a structure, which is also made probable by the X-ray analysis of crystalline glutamic acid [Bernal, 1931], explains the ease with which pyrrolidonecarboxylic acid is formed from glutamic acid.

Note on dissociation constants of proteins.

The work described here and in an earlier paper was undertaken partly with the intention of eliciting more information about the relationship of structure and ionization in complex compounds. Proteins are polyvalent electrolytes which according to currently accepted ideas consist of long peptide chains. Their ionization depends mainly on groups derived from trivalent amino-acids, such as the dicarboxylic and the basic amino-acids. It is commonly assumed, in the absence of any decisive evidence, that the γ-carboxyl groups of glutamic acid and the β-carboxyl groups of aspartic acid which are not present in amide linkage are free, whilst the corresponding α-carboxyl groups are in peptide linkage. If these assumptions are accepted, it is possible to predict with some accuracy the order of magnitude of the dissociation constants of the different groups. Effects through the chain of the molecule can be neglected if the substituent is more than 4 carbon atoms removed from the ionizing group, whilst charge effects depend entirely on the absolute distance and not on the distance in the direction of the chain and decrease generally more slowly with distance than do chain effects. A γ-carboxyl group on which no charge effects are acting will have *pK* 4·5–4·6, whilst a β-carboxyl group will have *pK* about 4·4–4·5. A protein molecule even in its isoelectric state carries a great number of positive and negative charges and the actual *pK* of any group will depend mainly on the distribution of positive and negative charges in its neighbourhood. If, for example, a carboxyl group is surrounded by a number of positive charges its *pK* will be much lower than 4·5. Since, in general, dissociation of carboxyl groups occurs over a *p*H range, where the number of positive charges of the protein molecule is larger than that of negative charges the average *pK* of carboxyl groups will be $<4{\cdot}5$. Proteins cannot be regarded as symmetrical polybasic acids; they are on the contrary highly unsymmetrical and the different strengths of the dissociating groups will be mainly responsible for the spreading of the titration curve; statistical factors will generally be of secondary importance. Only in the case of highly symmetrical proteins such as protamines will the spreading of dissociation constants be due to statistical factors. Since the charge effect is of such quantitative importance, a change in the shape of the molecule without any chemical alteration will cause a change in the individual dissociation constants and a shift of the isoelectric point since the distribution of charges will be altered. This may be the possible explanation of the experiments of Margaria [1934], who found that frog's muscle changed its apparent *p*H as measured by indicators during contraction and stretching.

All the methods available at present give somewhat questionable data about dissociation constants of proteins; but any discussion regarding this point is outside the scope of this paper.

SUMMARY.

1. α-Ethyl hydrogen glutamate has been synthesized.

2. Dissociation constants of glutamic acid, of α- and γ-ethyl hydrogen glutamate and of ethyl glutamate have been determined by extrapolating the apparent constants to zero ionic strength. Special formulae have been derived

to accommodate the effect of overlapping in the case of pK_1 and pK_2 of glutamic acid.

3. The different dissociation constants have been quantitatively explained on the basis of the corresponding structures; in particular it has been shown that the distance between NH_3^+ and the γ-carboxyl group in glutamic acid is smaller than the distance between the two carboxyl groups.

4. Principles derived from work on amino-acids have been considered in their application to proteins; it has been shown that dissociation constants and isoelectric points do not depend only on the chemical structure, but also on the shape of the molecules. Further, it has been pointed out that variation of dissociation constants in proteins depends on constitutional rather than on statistical factors.

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CCXCIV. THE MALIC DEHYDROGENASE OF ANIMAL TISSUES.

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THE following contribution is the fifth of a series devoted to the detailed analysis of individual dehydrogenase systems [cf. Ogston & Green, 1935, 1, 2; Green, 1936; Green & Brosteaux, 1936]. The immediate goal of these studies is a knowledge of the components of the various dehydrogenase systems, and an understanding of the mechanism by which these systems react with molecular oxygen. The ultimate goal is the reconstruction *in vitro* of series of parallel and coupled oxidations of the type living cells perform. The means by which the living cell integrates and co-ordinates the various oxidation systems has yet to be discovered.

All of the known dehydrogenases may be divided into three groups, viz. aerobic oxidases, cytochrome systems and coenzyme systems. Keilin & Hartree [1936] showed that the aerobic oxidases as a class react directly with molecular oxygen without requiring either a carrier or a coenzyme and produce H_2O_2 which can be utilized in coupled oxidations. The aerobic oxidases of animal tissues, e.g. uricase, amino-acid oxidase and xanthine oxidase are not generally distributed among the various organs and tissues but are restricted almost entirely to the liver and kidney. The general role of this class of oxidizing enzymes in cellular respiration must be therefore a minor one.

Ogston & Green [1935, 1, 2] and Green [1936] found that in animal tissues only the succinic and α-glycerophosphoric dehydrogenases depend upon cytochrome for their reaction with molecular oxygen. These two systems are found in all tissues in very considerable concentration. They do not require a coenzyme and do not react either with flavin or flavoprotein. The cytochrome catalysis involves the collaboration of two enzymes, the dehydrogenase and the indophenol oxidase. The former catalyses the reduction of cytochrome; the latter catalyses the oxidation of cytochrome by molecular oxygen. It is significant that the succinic and α-glycerophosphoric dehydrogenases as well as the indophenol oxidase are associated with particles which cannot be brought into solution.

The work of Warburg, Euler, Adler, Wagner-Jauregg and Green has made clear the characteristics of coenzyme dehydrogenase systems. This class of dehydrogenases is omnipresent in animal tissues, and there is good evidence that it accounts for the bulk of cellular respiration. Coenzyme dehydrogenase systems do not react directly with molecular oxygen. Between the substrate and oxygen, a coenzyme and a carrier intervene. The substrate in presence of the dehydrogenase reduces the coenzyme, reduced coenzyme reduces the carrier and reduced carrier then reacts with molecular oxygen. Flavin, flavoprotein or adrenaline can act *in vitro* as carriers in coenzyme systems, whereas cytochrome is completely inactive. Whether these carriers function physiologically has yet to be determined. There are only two coenzymes known to exist in animal tissues, viz. coenzyme I originally discovered by Harden & Young [1906] in yeast, and coenzyme II discovered by Warburg & Christian [1931] in horse blood corpuscles. They cannot replace one another in their respective catalytic capacities (with possibly one exception) despite their close similarity in chemical structure.

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The malic dehydrogenase offers a clear example of a coenzyme I system. Its properties are very similar to those of the lactic dehydrogenase—the prototype of the class. There is an extensive literature dealing with the malic dehydrogenase but little or no progress has been made in the way of isolating the enzyme and analysing the conditions and mechanism of the oxidation.

I. *Nomenclature.*

The great diversity of terminologies now in use among different investigators in the field of cellular respiration has rendered the literature almost unintelligible to the non-specialist. To avoid further confusion, the following list of synonymous terms is given—the preferred term being italicized.

Dehydrogenase: intermediary enzyme; colloidal carrier; oxidase; dehydrase; apodehydrase.

Flavoprotein: yellow enzyme; yellow pigment.

Coenzyme I: cozymase; coferment; fermentation coenzyme; codehydrase; lactic coenzyme; Harden & Euler coenzyme.

Coenzyme II: blood coenzyme; hexosemonophosphate coenzyme; coferment; codehydrase; Warburg coenzyme.

Coenzyme-dehydrogenase complex: holodehydrase; hydrogen transporting enzyme.

Carrier: this term refers to a substance which can be reversibly reduced and oxidised, regardless of chemical constitution or molecular dimensions. Thus cytochrome and flavoprotein as well as flavin and adrenaline are considered as carriers. The term should not be confused with colloidal carrier.

The practice of referring to the two oxidation coenzymes by numerals I and II was originally suggested by Euler & Adler. Its general acceptance is to be highly recommended.

II. *Preparation of the components.*

The malic dehydrogenase was prepared from the heart muscle of pig by the same method as was used by Green & Brosteaux [1936] for the preparation of the lactic enzyme. Since the concentration of malic enzyme in the heart muscle is extremely high, only two hearts need be used for the preparation of 300 ml. of very active enzyme solution (i.e. 100 ml. of solution A and 200 ml. of solution B). The Q_{O_2} of freshly prepared solution A or B varies from 300 to 800.

Coenzyme I was prepared from baker's yeast by the method of Green & Brosteaux [1936].

III. *The condition for linear oxidation.*

A mixture of dehydrogenase, coenzyme I, malate and methylene blue hardly takes up any oxygen. With addition of neutralized cyanide the oxygen uptake is rapid and linear for a considerable period, cf. Fig. 1. The maximum acceleration by cyanide is reached only at high concentrations. The control with cyanide and all components except malate is negative. There can be no doubt that the action of cyanide consists in reacting with the product of the reaction, presumably oxaloacetate—thereby preventing the complete inhibition of the enzyme by small concentrations of the reaction product. The fact that the cyanide effect is dependent upon the concentration indicates that the reaction of cyanide and oxaloacetate is not complete at low concentrations of the former.[1] The effect of cyanide on the malic system is exactly the same as that observed by Green & Brosteaux with the lactic system.

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[1] Smythe [1932] found that cyanide catalyses the anaerobic decomposition and polymerization of methylglyoxal. The possibility therefore exists that the action of cyanide consists not in combining with oxaloacetic acid but in catalysing the conversion of oxaloacetic acid into other products.

Other ketone reagents such as semicarbazide, hydrazine and hydroxylamine can be used instead of cyanide (cf. Table I).

Table I. *The effect of ketone fixatives.*

	$\mu l.\ O_2$	Time in min.
1. Enzyme system without fixative	25	60
+1·0 ml. *M*/2 semicarbazide (neutral)	214	60
+0·5 ml. *M*/2 semicarbazide	194	60
+0·2 ml. *M*/2 semicarbazide	147	60
2. Enzyme system without fixative	22	30
+1·0 ml. *M*/2 hydroxylamine (neutral)	35	30
+0·5 ml. *M*/2 hydroxylamine	52	30
+0·2 ml. *M*/2 hydroxylamine	57	30
3. Enzyme system without fixative	18	15
+1·0 ml. *M*/3 hydrazine (neutral)	141	15
+0·2 ml. *M*/3 hydrazine	94	15

The enzyme system comprised 0·5 ml. enzyme, 1 ml. coenzyme, 0·3 ml. 0·5% methylene blue and 0·2 ml. *M* malate. The total volume per manometer was 3·3 ml. The malate was tipped into the solution from a Keilin cup after equilibration of the manometer.

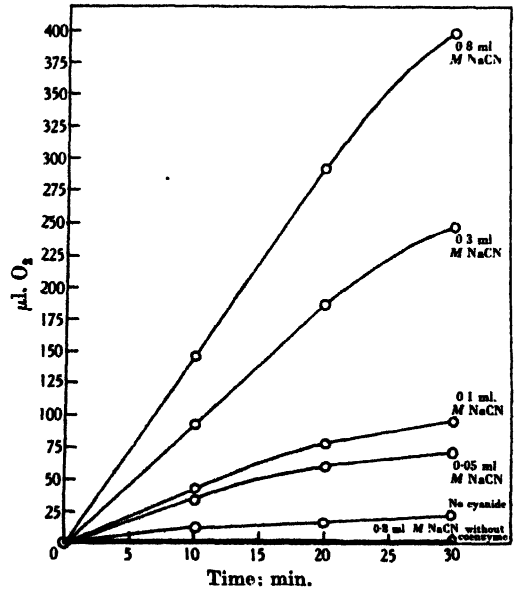

Fig. 1. The effect of cyanide. Each manometer cup contained 1·5 ml. enzyme, 0·5 ml. coenzyme, 0·2 ml. 0·5% methylene blue and 0·3 ml. *M* *l*(–)malate. The substrate was added from Keilin cups after equilibration.

The efficiency of a ketone-binding reagent in accelerating the oxidation depends upon three factors, viz. the toxicity to the enzyme, the speed of combination and the extent of dissociation of the addition compound. Cyanide is the most efficient reagent of those tested, with semicarbazide and hydrazine poor seconds.

IV. *The identity of the malic and fumaric dehydrogenase.*

Szent-Györgyi and co-workers [1935] have claimed that there exists in animal tissues a dehydrogenase specific for the oxidation of fumarate to oxaloacetate. The enzyme solution described in section II oxidizes both malate and fumarate at approximately equal speeds. The question arose whether the same enzyme catalyses both oxidations. The following lines of evidence show conclusively that the so-called fumaric dehydrogenase is simply the malic dehydrogenase collaborating with fumarase—the enzyme which catalyses the conversion of fumaric into malic acid.

(*a*) If the fumaric and malic enzymes are identical, then the ratio

$$\frac{\text{rate of oxidation of fumarate}}{\text{rate of oxidation of malate}}$$

should be 1 or less but never appreciably more. The actual value of the ratio will obviously depend upon the concentration of fumarase. With a small amount of this enzyme present compared with the malic enzyme, the ratio should be very much less than 1. With excess of fumarase, the ratio should be approximately 1.

Table II is a summary of the ratios observed in a large number of preparations of different degrees of purity and from a variety of tissues and animals. The evidence is clear that the ratio never exceeds 1:3 and is usually much lower.

Table II. *The ratio* $\frac{\textit{rate of oxidation of fumarate}}{\textit{rate of oxidation of malate}}$ *in different preparations.*

Source of enzyme	Time of experiment in min.	Ratio
Pig heart, solution A	20	1·18
Pig heart, solution B	60	1·27
Pig heart, solution B (purified)	60	1·20
Pig heart, solution A (purified)	20	0·48
Pig heart, solution A (purified)	20	0·45
Rabbit skeletal muscle	30	0·77
Rabbit kidney	20	1·06
Rabbit liver	10	0·45
Rabbit heart	15	0·78
Rabbit brain	10	0·39
Rat kidney	20	1·20
Rat heart	20	1·11
Rat muscle	60	0·43
Rat liver	60	1·04
Pigeon breast muscle	60	0·73

Furthermore, on purification it is always the fumaric activity which is lost relatively to the malic and never the reverse. The presence of active fumarase in the stock enzyme solution from pig's heart was established by the polarimetric demonstration of large quantities of malic acid formed from added fumaric acid. 1 ml. of enzyme was able to convert 1 mg. of fumaric into malic in 1 min. In other words, the limiting factor in the oxidation of fumaric acid in unpurified preparations is not the conversion into malic but the oxidation of malic. On purification fumarase activity is relatively diminished, and the conversion of fumaric into malic becomes the limiting factor.

(*b*) If the malic and fumaric enzymes are identical, there should be no summation of the rates of oxidation in presence of malate and fumarate respectively. The following experiment shows that such is actually the case.

	μl. O_2	
	1st 10 min.	2nd 10 min.
Enzyme system + malate	344	210
Enzyme system + fumarate	119	181
Enzyme system + malate + fumarate	328	230

(*c*) The oxidation of fumarate, particularly with purified preparations, shows an initial lag period varying from a few minutes to as much as an hour (cf. Fig. 2).

This phenomenon can be explained satisfactorily only on the basis that the oxidation of malate does not proceed at an appreciable velocity until the malate reaches a certain minimum concentration, and that the lag period represents the time required for a weak fumarase to convert sufficient fumaric into malic acid. It is significant that after the lag period, the rate of oxidation of fumarate attains the value for the rate of oxidation of malate.

There are many other lines of evidence which could be adduced to prove the identity of the two dehydrogenases, such as similar behaviour towards inhibiting and accelerating agents, similar kinetics, etc. But they are not as conclusive as those mentioned in detail and will therefore be omitted from the argument in the interest of brevity.

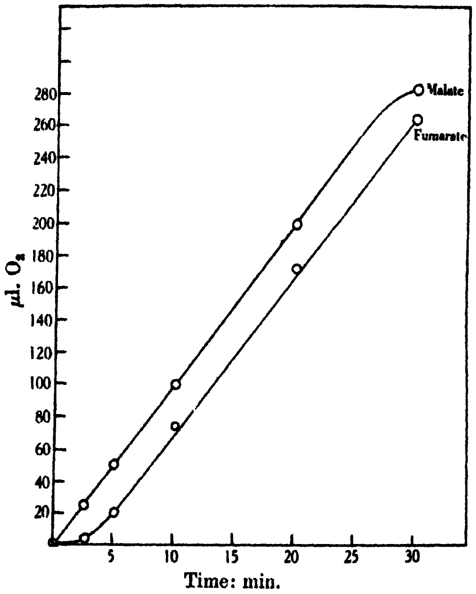

Fig. 2.

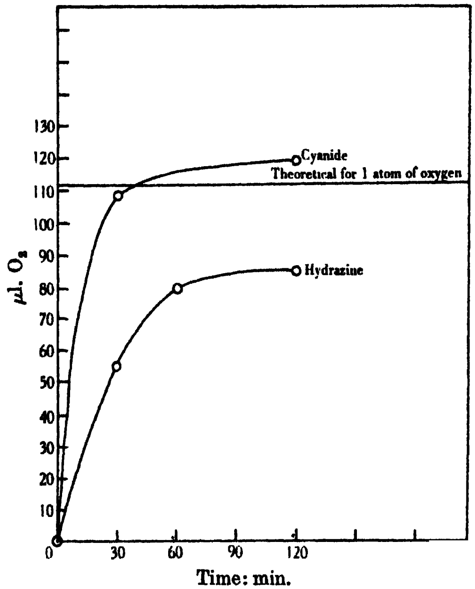

Fig. 3.

Fig. 2. The lag period in the oxidation of fumarate. Each manometer cup contained 1 ml. enzyme, 0·5 ml. coenzyme, 0·1 ml. 0·5 % methylene blue and 0·5 ml. *M* NaCN (neutralized).

Fig. 3. The number of oxygen atoms. Each manometer cup contained 0·8 ml. enzyme, 0·8 ml. coenzyme, 0·2 ml. 0·5 % methylene blue and 0·4 ml. *M*/40 *l*(–)malate. The concentrations of fixative were 0·5 ml. *M* NaCN and 1 ml. *M*/3 hydrazine respectively. The small control uptakes for the above mixtures without malate have been subtracted from the experimental uptakes.

V. *Product of oxidation.*

2:4-Dinitrophenylhydrazine does not react with oxaloacetic acid in presence of cyanide. The isolation was therefore carried out with semicarbazide as the fixing agent. 200 ml. of enzyme, 100 ml. of coenzyme, 20 ml. of *M* malate, 50 ml. of *M*/2 semicarbazide chloride (neutral) and 10 ml. of 0·5 % methylene blue solution were mixed and aerated vigorously at 37° for 2 hours. After deproteinization with hydrochloric acid and filtration through kieselguhr-impregnated filter-paper to remove methylene blue, the solution was concentrated *in vacuo* to 100 ml. 1 g. of 2:4-dinitrophenylhydrazine dissolved in 100 ml. of 2*N* HCl was added. The solution was kept at room temperature for 24 hours before filtering off the crystalline precipitate (yield 0·5 g.). After two recrystallizations from a mixture of ethyl acetate and ligroin by the method of Clift & Cook [1932], the M.P. was found to be 212° which is identical with that of the authentic 2:4-dinitrophenylhydrazone of oxaloacetic acid. The mixed M.P. was also 212°. The following are the analytical figures: (Weiler). (C, 38·78; N, 18·12; H, 2·39 %. $C_{10}H_8O_8N_4$ requires C, 38·34; N, 17·90; H, 2·58 %.)

The oxidation of malic to oxaloacetic acid requires 1 atom of oxygen. Fig. 3 shows that in presence of cyanide as the fixative, the theoretical uptake of oxygen for the oxidation of 0·1 ml. $M/10$ malate to oxaloacetate is reached within 30 min., whereas with hydrazine as the fixing agent, only 75 % of the theoretical is reached before the enzyme is completely inactivated.

If the sole chemical change is the oxidation of malate to oxaloacetate, then for each atom of oxygen absorbed, 1 molecule of oxaloacetic should be formed. Oxaloacetic acid can be estimated manometrically as CO_2 by the method of Ostern [1933] which involves the splitting of oxaloacetic acid by aniline in acid solution. Oxaloacetic acid after incubation with cyanide is not decomposed by aniline. However the semicarbazone is quite readily decomposed at 37° and the CO_2 liberation is complete in 3 hours. The experiments were carried out as follows. The complete enzyme system (enzyme, coenzyme, methylene blue and semicarbazide) was equilibrated in the manometer before the malate was introduced from a dangling Keilin tube. The oxygen uptake over a period of several hours was then measured. The manometer cup was then disengaged from the manometer and 0·3 ml. glacial acetic acid was added to deproteinize the enzyme solution. The aniline-citrate mixture (1 part of aniline to 3 parts of 50 % citric acid) was placed in a second Keilin tube and introduced after re-equilibration. The following table shows that for each atom of oxygen absorbed, approximately 1 molecule of CO_2 is liberated.

	µl. O_2	µl. CO_2	Ratio $\frac{\text{µl. } CO_2}{\text{µl. } O_2}$
Control (no malate)	5	4	—
Determination 1	264	570	2·16
2	245	505	2·06
3	187	368	1·98

VI. p*H*.

Table III shows the dependence of the rate of oxidation upon the hydrogen ion concentration. The maximum rate is at *p*H 8. It is interesting that the oxidation is completely inhibited by a very slightly acid reaction (*p*H 6) whereas a very alkaline reaction (*p*H 10–13) does not seriously interfere. The constituents of the buffer are probably as important as the *p*H in determining the speed of oxidation.

Table III. *The effect of* p*H*.

Each manometer cup contained 0·5 ml. enzyme suspension, 3·3 ml. of $M/2$ buffer, 0·5 ml. M NaCN, 0·1 ml. M fumarate, 0·2 ml. 0·5 % methylene blue and 0·5 ml. coenzyme. The enzyme suspension was made with water rather than the usual phosphate.

*p*H ...	6·0	7·0	8·0	9·0	10·0	11·0	12·0	13·0
Buffer ...	Phosphate	Phosphate	Phosphate	Glycine	Glycine	Glycine	Glycine	Glycine
				µl. O_2				
15 min.	0	37·8	84·0	37·5	15·4	29·0	20·5	17·0
30 min.	6·0	87·5	196·0	97·0	34·0	54·0	77·5	37·2

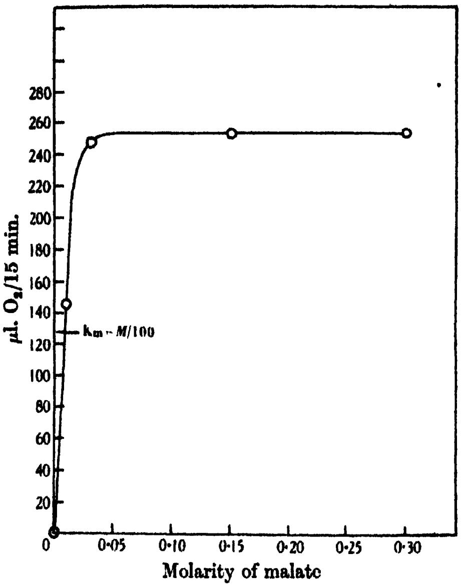

Fig. 4.

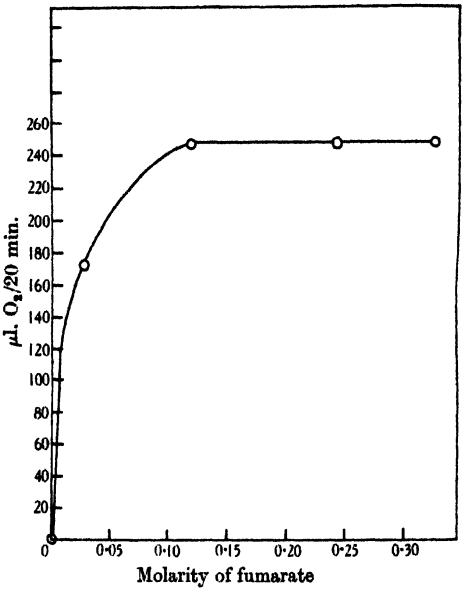

Fig. 5.

Fig. 4. The effect of fumarate concentration. Each manometer cup contained 1 ml. enzyme, 0·5 ml. coenzyme, 0·1 ml. 0·5% methylene blue and 0·5 ml. *M* NaCN.

Fig. 5. The effect of malate concentration. Each manometer cup contained 0·7 ml. enzyme, 0·5 ml. coenzyme, 0·7 ml. *M* NaCN and 0·1 ml. 0·5% methylene blue.

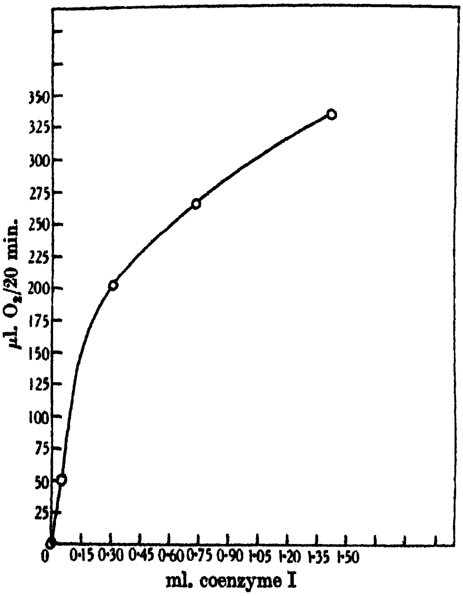

Fig. 6. The effect of coenzyme I concentration. Each manometer cup contained 1 ml. enzyme, 0·3 ml. *M* fumarate, 0·5 ml. *M* NaCN and 0·1 ml. 0·5% methylene blue.

VII. *Effect of concentration of substrate and coenzyme.*

The relation between the speed of oxidation and the molarity of substrate is shown in Figs. 4 and 5. The two sets of experiments with fumarate and malate respectively were not performed with the same enzyme preparation. Hence the absolute velocities are not comparable.

The rate of oxidation is proportional to the concentration of coenzyme over a very wide range of concentrations (cf. Fig. 6). The maximum asymptotic velocity would be reached only in presence of an enormous excess of coenzyme.

VIII. *Specificity of donator.*

Only *l*(−)malate is oxidized by the dehydrogenase system as shown by the following experiment:

	μl. O_2/15 min.
Enzyme system + 0·5 ml. *M*/10 *l*(−) malate	180
Enzyme system + 0·5 ml. *M*/10 *d*(+) malate	0

The optical isomerides were prepared from inactive malic acid by the method of Dakin [1924] which depends on the different solubilities of the cinchonine salts. Inactive malic acid is oxidized to the extent of only 50 % as shown by the fact that it yields exactly half the theoretical uptake for complete oxidation. Fumaric acid is converted into *l*(−)malic acid—hence the oxidation to oxaloacetic acid is quantitative.

Green & Brosteaux [1936] considered the evidence for and against the identity of the lactic and malic dehydrogenases and concluded that the two enzymes were probably not identical. The correctness of this conclusion has been confirmed by an extensive comparison of the activities of the two enzymes in different preparations from animal sources. In many cases, a preparation extremely rich in the malic enzyme showed little or no lactic activity. The following results are illustrative:

	μl. O_2	
	Exp. 1	Exp. 2
Enzyme system + lactate	39	20
Enzyme system + malate	282	392

It is noteworthy that whereas the method of preparation in section II invariably yields a highly active malic enzyme, success with the lactic enzyme is not as constant. The freshness of the tissue, the thoroughness of grinding and other factors in the preparation are more important for lactic activity than for malic activity. There can be little doubt therefore that the lactic and malic enzymes are distinct.

The dehydrogenase system, in presence of unpurified maleic acid (commercial preparations), reacts vigorously with oxygen. It can be demonstrated that such preparations are rich in fumaric acid and that when precautions are taken to remove all traces of fumaric acid, maleic acid is not oxidized. Freshly distilled maleic anhydride is practically inactive with the enzyme system. Since the conversion of maleic into fumaric acid occurs to some extent at 37° it is of course impossible to obtain a completely negative result. Dihydroxymaleic acid is also not oxidized.

IX. *Respiratory carriers and the reaction with molecular oxygen.*

A mixture of enzyme, coenzyme, malate and cyanide does not react with molecular oxygen. Some reversibly reducible and oxidizable substance is required to link the dehydrogenase system with molecular oxygen. The malic system must therefore be considered as anaerobic in the sense that the reaction with oxygen is not direct.

Table IV contains a comparison of the efficiencies of the various carriers which were found to be active. It is noteworthy that the adrenaline effect has a lag period. The oxygen uptake in presence of adrenaline for the second 10 min. should be compared with the first 10 min. results for the other carriers. Adrenaline shows catalytic activity comparable with methylene blue and pyocyanine and about twice the activity of lactoflavin. The detailed analysis of the adrenaline effect will be considered in a separate communication.

Table IV. *The activity of various carriers.*

Enzyme system was composed of 1 ml. enzyme, 1·4 ml. coenzyme, 0·4 ml. $2M$ NaCN and 0·2 ml. M malate.

	μl. O_2	
	1st 10 min.	2nd 10 min.
Enzyme system	3	3
$+M/1000$ lactoflavin	123	119
$+M/1000$ adrenaline	44	247
$+M/1000$ methylene blue	348	226
$+M/1000$ pyocyanine	299	178

Figs. 7, 8, 9 and 10 show how the speed of oxidation depends upon the concentration of the carrier. It is impossible to determine the limiting velocity for the catalysis by flavin owing to its relative insolubility. Lactoflavin was added in the form of a homogeneous aqueous suspension which went into solution only on dilution with the other components.

Flavoprotein shows but slight activity as a carrier:

	μl. O_2 in 10 min.	Manometer gas
Enzyme system	0	Air
Enzyme system	3	Oxygen
+1 ml. 10% flavoprotein	22	Air
+1 ml. 10% flavoprotein	35	Oxygen
+0·2 ml. 0·5% methylene blue	231	Air

Green & Brosteaux [1936] also observed comparatively little effect with flavoprotein. The fact that increase of the partial pressure of oxygen leads to a larger uptake in presence of flavoprotein suggests that the reoxidation of leucoflavoprotein is slow under the conditions of the experiment. It is presumably the spontaneous oxidation of leuco-flavoprotein which is the limiting factor and not the enzymic reduction of the oxidized form.

The reduction of added heart cytochrome *c* by the malic enzyme system was tested for spectroscopically. There was not the slightest indication of the reduced bands of cytochrome when the oxidized form was added to the enzyme mixture under anaerobic conditions. As a control, succinate was added under the same conditions and the immediate reduction of cytochrome *c* could be seen even with the naked eye. This confirms the rule that coenzyme systems do not react directly with cytochrome.

Oxidized glutathione did not act as a carrier with the malic system. Chemical estimation showed that no reduction of GSSG to GSH took place.

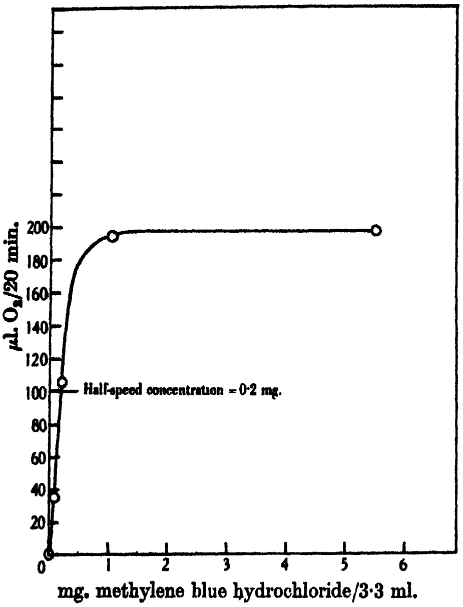

Fig. 7.

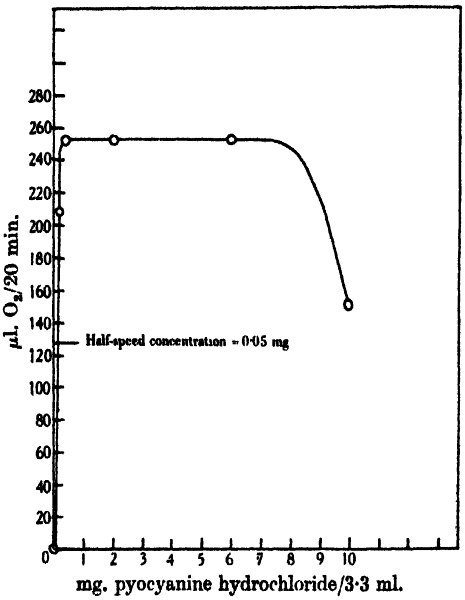

Fig. 8.

Fig. 7. The effect of methylene blue concentration. Each manometer cup contained 1 ml. enzyme, 0·5 ml. coenzyme, 0·5 ml. *M* NaCN and 0·3 ml. *M* fumarate.

Fig. 8. The effect of pyocyanine concentration. Details as for Fig. 7.

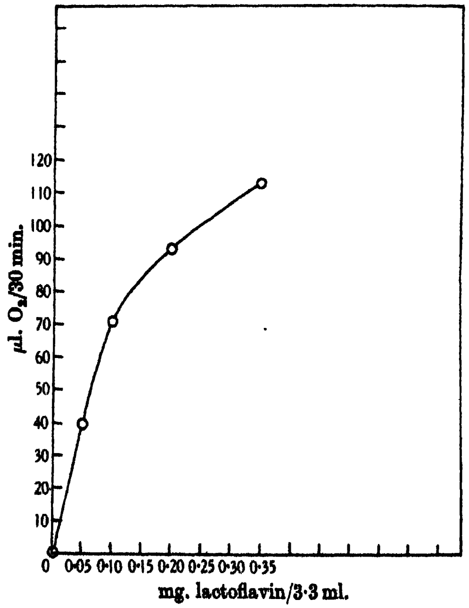

Fig. 9.

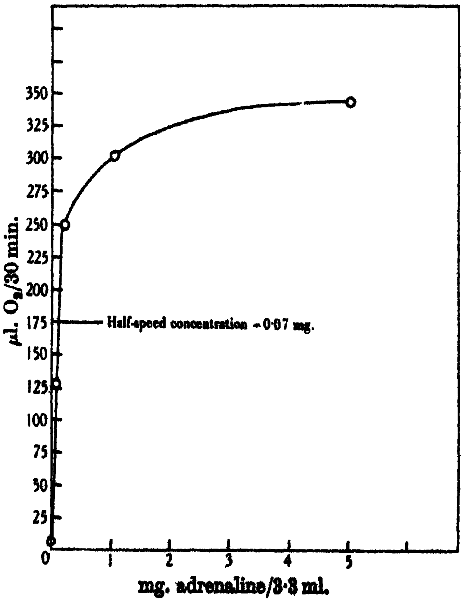

Fig. 10.

Fig. 9. The effect of lactoflavin concentration. Details as for Fig. 7.

Fig. 10. The effect of adrenaline concentration. Details as for Fig. 7.

X. *Reduced coenzyme and the mechanism of coenzyme action.*

Adler *et al.* [1936] and Green & Brosteaux [1936] independently demonstrated that coenzyme I, like coenzyme II, was reversibly reducible. Both groups of investigators agree on the main properties of reduced coenzyme such as insensitivity to strong alkali, sensitivity to acid, an absorption band in the ultraviolet with a peak at 340 mμ, rapid reaction with flavoprotein etc. The one important point of difference is the question whether the colourless reduced coenzyme can reduce methylene blue directly. Adler *et al.* claim that colourless reduced coenzyme can reduce methylene blue in acid but not in neutral solution. They describe the formation of a yellow substance by reduction of the coenzyme with hydrosulphite in alkaline solution. This substance, which is different from the colourless reduced coenzyme, can reduce methylene blue directly in alkaline solution. Green & Brosteaux reduced coenzyme I with hydrosulphite in neutral solution and showed with the vacuum tube technique that the solution of reduced coenzyme after removal of excess hydrosulphite by aeration could reduce methylene blue directly, albeit rather slowly. The addition of a small amount of flavoprotein increased the rate of reduction of methylene blue as much as thirtyfold. Hydrosulphite is oxidized to bisulphite which can reduce methylene blue. Controls however showed that the bisulphite present in solutions of reduced coenzyme could not account for the velocity with which methylene blue is reduced.

The controlled experiment was carried out as follows. 15 ml. of alkali-inactivated coenzyme and 15 ml. of untreated coenzyme were each mixed with 3 ml. *M*/5 phosphate buffer *p*H 7 and 1 ml. of a 0·5 % hydrosulphite solution. After 10 min. incubation at 37°, both solutions were aerated vigorously for 20 min. The methylene blue reductions were carried out in Thunberg tubes, each tube containing 0·3 ml. 0·02 % methylene blue, 1 ml. buffer *p*H 7 and 1 ml. of coenzyme solution:

	Time of reduction in min.
Alkali-inactivated coenzyme	>120
Alkali-inactivated coenzyme + 0·3 ml. 10 % flavoprotein	>120
Coenzyme	30
Coenzyme + 0·3 ml. 10 % flavoprotein	1

The rate of reduction of methylene blue by reduced coenzyme depends upon the concentration of methylene blue, e.g. 1 ml. of reduced coenzyme reduced completely 0·1 ml. of 0·05 % methylene blue in 40 min. whereas it reduced ten times that quantity of methylene blue to the extent of 50 % in the same time.

Oxidizing agents such as I_2 and H_2O_2 destroy the reduced coenzyme. No way has yet been found of oxidizing bisulphite to sulphate without destroying the reduced coenzyme at the same time.

Green & Brosteaux pointed out that the rate of reduction of methylene blue by reduced coenzyme even in presence of flavoprotein was very much slower than the reduction of methylene blue by the enzyme-coenzyme-lactate system. This discrepancy is difficult to explain on the basis of the hypothesis that the reaction between the coenzyme and the carrier does not involve the enzyme. In the present work other difficulties have been found in the way of applying the new conception of the mechanism of coenzyme action to the case of coenzyme I.

One would expect that the properties of reduced coenzyme would be the same regardless of the mode of reduction. But in fact reduced coenzyme prepared by incubation with the enzyme and the substrate under anaerobic conditions shows only in a qualitative way the properties of reduced coenzyme prepared by the hydrosulphite method. Whereas 1 ml. of the former can reduce 0·3 ml. of 0·02 % methylene blue in presence of flavoprotein within a minute, 1 ml. of the latter requires 30 min. or more. It is conceivable, as Euler and his group believe, that there is an equilibrium between reduced and oxidized coenzyme on the one hand and the reductant and oxidant of the enzyme system on the other—the equilibrium being in favour of the oxidized coenzyme. But this explanation cannot hold when a fixative like cyanide or semicarbazide is used to remove the oxidant. No increase in reducing power is obtained when the coenzyme is incubated with the enzyme, malate and fixing agent and thereby reduced to completion.

It remains for future research to determine whether the enzyme is involved in the reaction between the coenzyme and the carrier and whether coenzyme systems are carrier-specific. There can be no disputing the fact that the coenzyme is reversibly reduced and oxidized. But our knowledge of the mechanism of the interaction of reduced coenzyme with the other components in the catalytic system is still obscure.

XI. *The dismutation of fumarate.*

In the course of an attempt to study the reversibility of the oxidation of malic to oxaloacetic acid, it was observed that indicators as far apart in potential as methylene blue and flavin were not reduced to completion. The phenomenon therefore could not be attributed to an oxidation-reduction equilibrium. Analysis showed that the following series of events took place:

$$\text{(1) Malate} \xrightleftharpoons{\text{fumarase}} \text{fumarate.}$$

$$\text{(2) Malate} + \text{methylene blue} \xrightarrow[\text{enzyme}]{\text{malic}} \text{oxaloacetate} + \text{leuco-methylene blue.}$$

$$\text{(3) Fumarate} + \text{leuco-methylene blue} \xrightarrow[\text{enzyme}]{\text{succinic}} \text{succinate} + \text{methylene blue.}$$

The indicator, methylene blue in this case, is linking two dehydrogenases, the succinic and the malic; and is being oxidized by the former and reduced by the latter. The phenomenon of partial reduction therefore offers an interesting example of linked carrier reactions between dehydrogenase systems [Green *et al.*, 1934]. Reduction is never complete since fumarate is oxidizing leuco-methylene blue at a rate comparable with the reduction of methylene blue by malate.

The proof of this interpretation lies in the demonstration of the anaerobic formation of oxaloacetic acid from fumaric acid which had been incubated with the enzyme, coenzyme, methylene blue and semicarbazide. Controls lacking fumaric acid and methylene blue were negative. There was a small formation of oxaloacetate in presence of fumarate and with no coenzyme. The oxaloacetate was determined manometrically by the method of Ostern [1933]. Table V shows the results of an experiment.

Table V. *The anaerobic formation of oxaloacetate from fumarate.*

The experiments were carried out anaerobically in Thunberg tubes. Each tube contained 1 ml. of $M/2$ semicarbazide in addition to the solutions stated. The total volume in all cases was 7·7 ml.

Enzyme ml.	Coenzyme ml.	Methylene blue (0·5 %) ml.	Fumarate M ml.	Oxaloacetate mg.
3	3	—	0·5	0
3	3	0·2	—	0
3	3	0·2	0·5	4·0

The succinic acid formed was demonstrated in the following way. After incubation, the enzyme mixture was deproteinized with 0·2 ml. glacial acetic acid and filtered. The filtrate was kept in a boiling water-bath for 5 min. It was then made alkaline to thymolphthalein with 6 *N* NaOH and again placed in a boiling water-bath for 5 min. This treatment in acid and alkali destroys all traces of the coenzyme whether it be in the reduced or oxidized form. The solution was carefully neutralized and 4·0 ml. were tested for succinic acid with 0·5 ml. enzyme preparation and 0·2 ml. 0·5% methylene blue. The pig's heart preparation is very rich in the succinic enzyme and is quite suitable as the source of the enzyme. Fumaric and malic acids are not oxidized under these conditions since no coenzyme is provided. Table VI contains the protocol of an experiment.

Table VI. *The anaerobic formation of succinic acid.*

1·18 mg. succinic acid are equivalent to 112 μl. O_2 since the oxidation of succinate to fumarate only involves 1 atom of oxygen.

Enzyme ml.	*M*/3 semi-carbazide ml.	Coenzyme ml.	*M* fumarate ml.	0·5% methylene blue ml.	Succinic acid formed mg.
3	1	2	0·2	0·1	3·2
3	1	2	0·2	0	0
3	1	2	0	0·1	0

The observation of Moyle [1924] that in muscle succinic acid accumulates under anaerobic conditions indicates that this anaerobic dismutation of fumaric acid is a physiological process and that some cellular carrier takes the place of methylene blue in the model system.

XII. *Specificity of coenzyme.*

Needham & Green in unpublished experiments quoted by Green & Brosteaux [1936] studied the question of the identity of the lactic coenzyme and cozymase. They concluded that although the parallelism of the occurrence and activity of the two coenzymes pointed to their identity the mechanism of the action of the coenzyme was not identical in both fermentation and dehydrogenation. The recent note of Meyerhof & Kiessling [1936] describing the isolation of cozymase-pyrophosphate from yeast maceration juice indicates that cozymase is concerned with phosphorylation as well as dehydrogenation. Such being the case, to call the lactic coenzyme, cozymase or *vice versa* is illogical. The suggestion by Euler & Adler of calling cozymase, coenzyme I overcomes this difficulty. Coenzyme I is to be understood as the coenzyme which functions either in alcoholic fermentation or in certain dehydrogenations.

Coenzyme II was tested as an alternative coenzyme for the malic system. The following are the results.

	μl. O_2/40 min.
Enzyme system + 0·5 ml. coenzyme II (no malate)	5
Enzyme system + 0·5 ml. coenzyme II (malate added)	28
Enzyme system + 0·5 ml. coenzyme I (malate added)	258

The enzyme system contained 1 ml. enzyme, 0·2 ml. 0·5% methylene blue and 0·6 ml. *M* NaCN

The coenzyme II preparation used was about 30% pure and it is possible that traces of coenzyme I account for the slight effect on the malate oxidation. The point requires further study.

Trigonelline, nicotine, coramine, coramine methiodide and nicotine dimethiodide showed no activity as coenzyme. It seems highly probable that the nicotinamide molecule must be specifically linked with adenine, ribose and phosphoric acid in order to function as a coenzyme.

XIII. *Inhibitors.*

Table VII contains a summary of the effects of various reagents on the oxidation of malate. Oxaloacetic acid is the most powerful inhibitor found. Hence the indispensability of ketone-fixing reagents for studying the *in vitro* oxidation of malic acid. Pyruvic acid and acetoacetic acid are inhibitory but not to as high a degree as oxaloacetic. Animal adenylic acid and adenylpyrophosphate inhibit presumably by competing with the coenzyme for the dehydrogenase. Yeast adenylic acid is much less inhibitory. Maleic acid contrary to the statements in the literature does not act as an inhibitor. The mechanism of the acceleration by arsenious acid and pyrophosphate is obscure.

Table VII. *Inhibitors.*

Reagent	Concentration *M*	% inhibition	% acceleration
Oxaloacetic acid	0·18	100	—
	0·09	100	—
	0·018	96	—
Acetoacetic acid	0·3	39	—
	0·15	21	—
	0·03	0	—
Pyruvic acid	0·3	92	—
	0·06	76	—
	0·012	24	—
Muscle adenylic acid	0·0087	49	—
	0·0044	23	—
	0·00087	13	—
Yeast adenylic acid	0·0087	16	—
	0·0044	0	—
Adenylpyrophosphoric acid	0·0034	40	—
	0·0017	22	—
Iodoacetic acid	0·03	38	—
Arsenious acid	0·03	—	38
Pyrophosphoric acid	0·03	—	104
Urethane	0·017	10	—
Tartronic acid	0·06	24	—
Capryl alcohol	Saturated solution	18	—
Malonic acid	0·3	47	—
	0·03	11	—
Oxalic acid	0·075	30	—
Maleic acid	0·03	0	—

There is a considerable uptake by oxaloacetic acid solutions (non-enzymic) which must be allowed for in calculating the inhibition by oxaloacetic acid.

All the inhibitor experiments were carried out with the enzyme system (enzyme, coenzyme, malate and methylene blue) in presence of cyanide as the fixative. Since oxaloacetic, acetoacetic and pyruvic acids combine with cyanide, the effective concentrations involved in the respective inhibitions must have been at least ten times as small as the added concentrations. Without cyanide, the oxygen uptake is not appreciable: hence inhibition experiments are not possible without the use of fixing agents. It is possible, however, to calculate the actual inhibitory power of oxaloacetic acid from the limiting oxygen uptake of the enzyme system in absence of cyanide. Calculation shows that $M/1000$ oxaloacetic acid poisons the enzyme completely.

Table VIII. *The distribution of the malic dehydrogenase.*

Q_{O_2} malic dehydrogenase is the oxygen uptake in μl./hour/mg. dry weight of enzyme solution. The dry weight of the phosphate buffer is subtracted from the total dry weight.

	Q_{O_2} malic dehydrogenase		
	Rat	Rabbit	Pigeon
Brain	60·0	55·1	72·2
Kidney	10·8	23·0	124
Liver	40·6	29·4	60·5
Heart	218·0	56·7	189
Muscle	81·5	3·6	174

XIV. *Distribution of the enzyme.*

The method of estimation of the concentration of malic enzyme in various tissues is more or less that described by Green & Brosteaux [1936]. The tissue is minced finely with scissors and thoroughly washed with water. The last two washings are carried out with $M/50$ phosphate buffer of *p*H 7·2. The brei is then thoroughly ground with sand and $M/50$ phosphate buffer. After centrifuging the supernatant is tested directly for activity with 1 ml. coenzyme, 0·2 ml. 0·5 % methylene blue, 0·4 ml. $2M$ NaCN and 0·2 ml. M malate. The results are shown in Table VIII. The malic dehydrogenase is found in all tissues in extremely high concentration, particularly in heart. The chemical pathway leading from succinic to oxaloacetic acid must be of great importance in the intermediary metabolism of the various tissues.

SUMMARY.

The preparation of a highly active malic dehydrogenase from the heart muscle of the pig is described.

The product of oxidation, oxaloacetic acid, completely inhibits the oxidation even in extremely small concentration. The use of ketone fixatives such as cyanide, hydrazine, semicarbazide and hydroxylamine is essential in order to obtain a linear oxidation.

The catalytic system comprises the dehydrogenase, coenzyme I, carrier and malate. Methylene blue, pyocyanine, lactoflavin and adrenaline are the most active carriers, flavoprotein is only slightly active whereas cytochrome and glutathione are inactive.

The enzyme system specifically oxidizes *l*(−)malic acid to oxaloacetic acid—the latter being isolated as the 2:4-dinitrophenylhydrazone.

The so-called fumaric dehydrogenase is merely malic dehydrogenase collaborating with fumarase.

The malic enzyme is not identical with the lactic enzyme.

Fumaric acid has been shown to dismute anaerobically to form succinic and oxaloacetic acids. The dismutation depends upon the presence of the succinic and malic enzymes, coenzyme I and a suitable carrier.

The malic dehydrogenase is found in high concentration in the tissues of the rat, rabbit and pigeon.

It is a pleasure to thank Mr Stanley Williamson for his kind assistance with many of the chemical preparations. I am also grateful to Prof. Richard Kuhn for a gift of lactoflavin and to Messrs Ciba, Ltd., for a gift of coramine.

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CCXCV. THE BIOLOGICAL SPLITTING OF CONJUGATED BILE ACIDS.

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The biological splitting of the conjugated bile acids into their two components, amino-acid and cholic acid, is important both from the chemical and physiological points of view. Two possible ways of effecting this reaction have to be considered: (1) by means of tissue enzymes; (2) by bacteria and moulds.

Several authors have investigated this problem but their results are of a somewhat contradictory nature.

The present work was undertaken with the intention of clarifying the position.

I. The question of the splitting of conjugated bile acids by enzymes from animal organs.

Smorodinzew [1923] in the course of his work on histozyme, the classical enzyme splitting hippuric acid [cf. Mutch, 1912], found that horse and canine kidney extracts which were rich in histozyme could also split glycocholic and taurocholic acids, and that preparations of dog liver, of a somewhat feebler activity against hippuric acid, hydrolysed taurocholic acid but scarcely attacked glycocholic acid. From his results he inferred that histozyme is able to split not only hippuric acid but also the conjugated bile acids. His experiments which were carried out with a rather primitive technique do not allow of more than qualitative conclusions.

Domenico [1926] working with liver pulp of various mammals did not observe splitting of the conjugated bile acids and concluded that the liver of mammals does not contain a specific enzyme capable of hydrolysing the bile constituents mentioned. Rosenthal *et al.* [1927] working with pulp of human liver and spleen state that they observed splitting of taurocholic acid after incubation for 5–6 days. Grassmann & Basu [1931] employing a modern technique confirmed the statements of Smorodinzew as regards the observed ability of kidney extracts to hydrolyse (although only to a rather limited degree) taurocholic and glycocholic acids; they did not however succeed in hydrolysing these acids by the action of liver extracts at *p*H 8.

On the other hand Mazza & Stolfi [1932] claimed to have found regularly in liver extracts of different animals a specific enzyme which hydrolysed the conjugated bile acids quickly and synthesized them from their two components. They attribute Grassmann's failure to observe this enzyme to the fact that it is only active in a weakly acid or neutral medium (*p*H optimum being 6·0); whereas Grassmann & Basu were working at *p*H 8, at which the enzymic action according to Mazza & Stolfi is inhibited. The latter suppose that the enzyme found by them to be regularly present in the liver is different from histozyme, though they agree with previous workers that the histozyme of the kidney is also capable of splitting the conjugated bile acids. They do not seem to have investigated however whether their specific enzyme does not split hippuric acid as well.

There seems to be general agreement among the different workers on the following points.

(1) Enzyme preparations from the pancreas and from the mucosa of the duodenum do not split the conjugated bile acids.

(2) Kidney extract which contains histozyme also hydrolyses the conjugated bile acids. This action is slow and feeble and probably has no physiological importance since the bile acids do not traverse this organ during their circulation.

As regards the activity of the liver, which from the physiological point of view is undoubtedly the most important organ in this respect, the above review reveals discrepancies in the findings of the several workers.

The experiments described below were undertaken in order to clear up this point. A comparative investigation of the activities of enzymic liver preparations upon both hippuric acid and the conjugated bile acids has been carried out for the first time.

Preparation and study of acetone-ether-dried liver extract.

Dried powder was prepared from dog liver according to Willstätter & Rohdewald [1934]. The dried material was finely ground and then sieved through a close-meshed sieve. Weighed amounts of the powder were used in the experiments. The buffer solutions employed were mixtures of citric acid ($M/10$) and Na_2HPO_4 ($M/15$). The substrates were pure hippuric acid neutralized with NaOH, and pure Na salts of glycocholic or taurocholic acid. The determinations were carried out by Sørensen's formaldehyde titration using $N/10$ NaOH, taking 4 ml. of the reaction mixture for each titration. The reaction temperature was 37°.

Enzyme experiments. (*a*) 0·4 g. hippuric acid dissolved and neutralized with dilute NaOH; 1 g. of liver preparation; 10 ml. buffer solution *p*H 7; water to 35 ml.; 5 ml. toluene.

(*b*) (I): 0·7 g. Na taurocholate; 0·5 g. liver preparation; 10 ml. buffer solution *p*H 7; 15 ml. water; 5 ml. toluene.

(II): like (I), except that the buffer solution was of *p*H 8.

(*c*) (I): like (*b*) (I), except that the substrate was Na glycocholate.

(II): like (*b*) (II), except that the substrate was Na glycocholate.

Controls: liver preparation + corresponding buffer solutions; substrates + corresponding buffer solutions. All controls have the same concentration as the corresponding enzyme experiments.

The figures given in Table I are the observed changes after the corresponding control values are deducted.

Table I. *Action of dog liver preparation (dried with acetone-ether) on hippuric, glycocholic and taurocholic acids.*

		Increase in ml. $N/10$ NaOH per 4 ml. reaction mixture after the indicated number of days											
	*p*H	1	2	3	4	5	6	7	8	9	10	11	12
Na hippurate	7	0·16 (6·3%)	0·36 (14·1%)	—	—	1·06 (41·5%)	—	—	—	—	—	—	1·96 (77%)
Na taurocholate	7	0	0	0·08	—	0·08	—	0·16	—	0·14	0·14	—	0·08
	8	0	0	—	0·0	—	−0·18	—	−0·18	—	—	—	—
Na glycocholate	7	0·04	—	0·06	0·08	—	0·06	—	—	—	0·06	—	—
	8	0·0	—	−0·10	—	−0·14	—	−0·14	—	—	—	—	—

Table I shows that the liver preparation contains an enzyme capable of splitting hippuric acid, i.e. the classical histozyme. Neither at *p*H 7 nor 8 were the conjugated bile acids split. The slight irregularities observed are due to the presence of small solid liver particles in the reaction mixtures and are within the experimental error. This result suggests (1) that the enzyme contained in this liver and splitting hippuric acid is not able to split taurocholic or glycocholic acid; (2) that it must therefore be different from that found by other authors in the kidneys which splits both hippuric and conjugated bile acids and (3) that this liver preparation does not contain the specific enzyme of Mazza & Stolfi capable of splitting taurocholic and glycocholic acids.

At the time these experiments were carried out the paper of the last-mentioned authors was unknown to us. Since the technique employed by these authors differed from that used by us in that they did not use the dried liver preparation directly but extracted it with glycerol, we repeated their experiments with fresh ox liver and made an acetone-ether-dried preparation as well as a direct glycerol liver extract according to the details given by Mazza & Stolfi. According to these authors both preparations should be active.

Preparation and study of glycerol liver extracts.

Liver of a freshly slaughtered ox was used for both preparations according to Mazza & Stolfi.

(*a*) *Glycerol liver extract* (*direct extraction*). The liver was roughly ground, minced and triturated with quartz in 90% glycerol in a porcelain mortar. The liquid was then filtered off or centrifuged.

(*b*) *Acetone-ether preparation and subsequent glycerol extraction.* The material was dried by acetone-ether treatment according to Willstätter & Rohdewald and was then extracted with glycerol-water mixture (containing 80% glycerol) at 37° during 24 hours.

The following substrates were used: (1) glycylglycine; (2) *dl*-leucylglycine; (3) hippuric acid; (4) Na glycocholate; (5) Na taurocholate. Buffer mixtures: the same as used by Mazza & Stolfi, i.e. phosphate buffers (*M*/15). Titrations according to Sørensen, employing 2 ml. of the reaction solutions and *N*/10 NaOH.

(A) *Enzymic experiments with glycerol liver extracts.* (1) 0·3 g. *dl*-leucylglycine; 8 ml. buffer *p*H 8; 5 ml. glycerol liver extract; water to 25 ml.

(2) 0·3 g. glycylglycine, all other components as in (1).

(3) 1·4 g. hippuric acid neutralized with *N* NaOH; 8 ml. buffer *p*H 7; 4 ml. liver extract; water to 25 ml.

(4) 1·5 g. Na glycocholate; 8 ml. buffer *p*H 6·2; 4 ml. liver extract; water to 25 ml.

(5) 1·5 g. Na taurocholate, all other components as in (4).

(B) *Experiments using glycerol extract of acetone-ether-dried liver.* (1) 0·3 g. glycylglycine; 8 ml. buffer *p*H 8·0; 5 ml. liver extract; water to 25 ml.

(2) 1·4 g. hippuric acid neutralized with *N* NaOH; 8 ml. buffer *p*H 7; 3 ml. liver extract; water to 25 ml.

(3) 1·5 g. Na glycocholate; 8 ml. buffer *p*H 6·2; 3 ml. liver extract; water to 25 ml.

In all cases described under A and B the appropriate controls were conducted, and the control values observed were deducted from the figures given in Table II. The reaction temperature throughout was 37°.

The results summarized in Table II are in accordance with those of Mazza & Stolfi as regards the presence of dipeptidase in both preparations but do not

Table II. *Action of glycerol ox liver extract preparations on dipeptides, hippuric acid and conjugated bile acids.*

		Increase in ml. $N/10$ NaOH per 2 ml. reaction mixture after the indicated number of hours								
Substrate	*p*H	1	2	3	19	20	48	68	96	168
Experiments with extract obtained by direct extraction of liver with glycerol.										
Glycylglycine	8	0·50	0·60	0·65	—	1·40	—	—	—	—
dl-Leucylglycine	8	0·22	0·30	—	0·46	0·48	—	0·54	—	—
Na hippurate	7	—	—	—	0·0	—	0·0	—	0·02	0·03
Na glycocholate	6·2	—	—	—	0·0	—	0·0	—	0·0	0·0
Na taurocholate	6·2	—	—	0·03	—	—	0·0	—	0·0	—
Experiments with extract obtained by previous treatment of liver with acetone-ether and subsequent extraction with glycerol.										
Glycylglycine	8	0·0	0·10	—	—	1·15	—	—	—	—
Na hippurate	7	0·0	—	—	0·0	—	0·0	—	0·0	0·04
Na glycocholate	6·2	0·0	0·0	—	—	—	0·0	—	0·0	0·0

support the findings of these authors as regards the presence also of a specific enzyme splitting the conjugated bile acids. The preparations hydrolysed *dl*-leucylglycine and glycylglycine but, as in the previous case with dog liver, failed to split glycocholic or taurocholic acid, although the experiments were carried out at the optimum *p*H or within the *p*H range given by Mazza & Stolfi for the activity of their specific enzyme. Thus no cholic acid could be detected by qualitative test in the reaction mixtures. Our results are in agreement with those of Domenico and Grassmann & Basu and moreover cover a broader *p*H range.

II. THE EXISTENCE OF BACTERIA CAPABLE OF HYDROLYSING CONJUGATED BILE ACIDS.

As early as 1876 Hoppe-Seyler supposed that taurocholic acid could be hydrolysed by bacteria [1876]. Since cholic acid is regularly found in the faeces, it has been assumed that bacterial hydrolysis of the conjugated bile acids occurs during their passage through the intestines. This has never been proved experimentally. Several authors [Licht, 1924; Rosenthal *et al.*, 1927], working mainly with pure strains of bacteria known to be present in the intestines, failed to find hydrolysis of the conjugated bile acids into their components; they supposed that the molecule of the bile acids was attacked by bacteria at other places, the .CONH. linkage between the two components remaining intact.

Isolation of bacteria splitting conjugated bile acids.

The bacteriological part of the following experiments was carried out by Dr M. Aschner.

A synthetic nutrient medium was used containing 1–2 % of either glycocholic or taurocholic acid as sole source of C and N, and the following inorganic salts: K_2HPO_4 (0·1 %), $MgSO_4$ (0·1 %), $CaCO_3$ (0·2 %) and traces of iron. The medium was inoculated with small amounts of different biological materials, such as soil, contents of the lower part of canine intestines, human faeces and in a few cases also human intestinal contents taken during autopsy. After keeping at about 20–25° under aerobic conditions in positive cases bacterial growth was observed. By re-inoculating into the same medium pure cultures of various strains were finally obtained, which, as will be shown, are able to hydrolyse glycocholic and taurocholic acids, liberating cholic acid.

The bacteria are Gram-negative, mobile and produce thick membranes and pigments in media containing bile acids. They do not grow *in vitro* at 37°. A detailed description of the bacteriological properties of the strains will be given elsewhere by Dr M. Aschner.

As regards moulds it should be noted that Grassmann & Basu [1931] found that an aqueous extract of *Aspergillus oryzae* splits glycocholic acid very slightly but taurocholic acid not at all. We have more than once observed growth of mould organisms on synthetic nutrient containing bile acids.

Detection and isolation of cholic acid liberated from conjugated bile acids by bacteria.

Pure Na taurocholate and glycocholate were used in these as well as in the enzymic experiments. Many commercial samples contain significant amounts of other substances which caused us much trouble until this source of complications was traced. In these experiments the nutrient medium contained besides the salts mentioned above 2 % of the conjugated bile acid. In order to avoid prolonged heating which favours hydrolysis of the taurocholic acid the solutions were sterilized by filtering through a Seitz filter. Solid $CaCO_3$ was afterwards added. To this solution was added a small amount of one of the previously mentioned pure cultures of bacteria grown on conjugated bile acid and washed when necessary. The solution was kept at 20–25° until bacterial growth was sufficiently developed. For the qualitative detection of cholic acid the Mylius reaction which is not given by the conjugated bile acids and is specific for cholic acid was used: some of the solution to be tested was acidified with *N* HCl, and after decanting the supernatant fluid the precipitate was dissolved in alcohol; iodine solution was added and then water drop by drop with shaking. In the presence of cholic acid, green-blue needles suddenly appear which often fill the whole test-tube as a voluminous fluorescent mass. In order to isolate the cholic acid, ether was added to the solution in a separating funnel and then, with continuous shaking, *N* HCl until acid to Congo red. Under such conditions cholic acid generally passes into the ether layer. Only when greater volumes are handled does some remain undissolved. The ether layer was dried with $CaCl_2$ and evaporated. The crystalline residue was dissolved in a little hot absolute alcohol and kept for some days in the ice-box with repeated scratching with a glass rod. The crystals were then collected, washed several times with ice-cooled alcohol and dried *in vacuo* at 100°. They gave all the reactions of cholic acid. In most cases crystallization and drying had to be repeated, before the melting point of pure cholic acid (197·5°, not corrected) was reached. Identity of this product with cholic acid was finally confirmed by mixed melting point.

Such experiments were carried out with both glycocholic and taurocholic acids on a number of strains isolated from the different sources indicated above: human faeces, human intestines, dog intestines and soil. The yields of cholic acid were relatively considerable. Since the operations of isolation and recrystallization involve considerable losses and also since the cholic acid seems to be used as a nutrient by the bacteria, no quantitative data are given. Hitherto it has not been possible to separate the enzyme from the bacterial cell.

As mentioned above the participation of the bacterial flora of the intestines in the process of splitting conjugated bile acids has been discussed several times in the past, although previous workers have not found such bacteria.

As regards the physiological interpretation of the present findings the following should be noted.

(1) It was not possible to isolate the bacteria described from every sample of human faeces.

(2) The bacteria described here do not grow at body temperature *in vitro*.

It was attempted to ascertain whether these bacteria are able to hydrolyse glycocholic and taurocholic acids at 37°. Cultures of strains from human and dog intestines and grown at about 25° were washed free of their medium until they did not give the test for cholic acid. They were then introduced into media containing either Na taurocholate or glycocholate and kept at 37° under sterile conditions, together with appropriate controls consisting of the same media without inoculation of the different strains. The controls remained negative. In inoculated mixtures it was possible within 24 hours to detect and, where it was attempted, also to isolate cholic acid.

It is therefore clear that the bacteria, although they do not grow at 37° *in vitro* are capable of hydrolysing the conjugated bile acids at this temperature.

SUMMARY.

1. A dog liver preparation capable of splitting hippuric acid did not hydrolyse the conjugated bile acids. Liver histozyme therefore does not necessarily split the conjugated bile acids.

2. No enzyme splitting the conjugated bile acids (or hippuric acid) was found in an ox liver preparation.

3. Bacteria were isolated from soil, human and dog intestines and from human faeces which grow on synthetic nutrient media containing conjugated bile acids as sole source of carbon and nitrogen and which split these acids into their two components.

4. Although not growing at 37° *in vitro* tested strains hydrolysed the conjugated bile acids at this temperature *in vitro*.

The author is indebted to Mr A. Levin, M.Sc., who collaborated with him in the experiments on dog liver.

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CCXCVI. THE THERMOCHEMISTRY OF THE OXYGEN-HAEMOGLOBIN REACTION.

II. COMPARISON OF THE HEAT AS MEASURED DIRECTLY ON PURIFIED HAEMOGLOBIN WITH THAT CALCULATED INDIRECTLY BY THE VAN'T HOFF ISOCHORE.

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INTRODUCTION.

In Part I [Roughton, 1935] data have been given for:

(A) The relation between the heat liberated and the percentage saturation of haemoglobin with oxygen when haemoglobin is equilibrated with insufficient oxygen to saturate it completely.

(B) The effect of laking and of purification on the heat of reaction of oxygen with haemoglobin.

(C) The relation between the heat of the reaction and the pH and buffer content of the solution. This question has also been treated theoretically.

(D) Furthermore Bateman & Roughton [1935] have shown that the "slow" heat of reaction between O_2 and Hb (as measured by the classical methods of calorimetry) agrees within experimental error with the "rapid" heat liberated in 0·008–0·02 sec.

These results all conform to simple expectations. The present paper deals with the last item of the programme, sketched in Part I, namely:

(E) The comparison, for purified haemoglobin solutions, of the heat of the reaction, as measured directly, with that calculated indirectly from the effect of temperature on the oxyhaemoglobin dissociation curve by means of the Van't Hoff Isochore, which runs [Adair, quoted by Barcroft, 1928]

$$Q_y' = \frac{RT_1T_2}{T_1 - T_2} \log_e \frac{C_1}{C_2} \qquad \text{......(1)},$$

where Q_y' = heat of reaction at percentage saturation y; R = the gas constant, i.e. 1·98 calories; C_1, C_2 are the respective concentrations of O_2 in the gas phase required to maintain the haemoglobin at percentage saturation y at absolute temperatures T_1, T_2 respectively.

Such a comparison (in the case of purified Hb) has not been made since the pioneer work of Barcroft & Hill [1909], in which, however, abnormally high values for the heat of reaction were obtained. To secure reliable data of this kind, requires preferably a team of workers, owing to the lability of the haemoglobin. In the present paper experimental data have been obtained under two different sets of conditions:

(I) At pH 6·8, $M/15$ phosphate buffer (by Goldschmidt, Ray & Roughton). In this region it was experimentally possible to get reliable data over a large range of temperature (0–40°) and thus to test equation (1) very thoroughly. In particular it was desired to check the view of previous workers, that the effect of temperature on the dissociation curve is to alter its scale, but not to alter its shape. It was also desired to see whether the effect of temperature could be expressed by a smooth relationship, or whether there were, at or near certain temperatures, breaks similar to those reported by Crozier [1924] for complex physiological processes.

The results were not, however, expected to be so clear-cut from the theoretical point of view, for within the *p*H range 6·0–9·0, the heat of reaction is affected by the dissociation and subsequent buffering of the oxy-labile H ions which are liberated when haemoglobin is oxygenated (for definition of this term and discussion, *v.* Roughton [1935]). Under these conditions an elaborate theoretical treatment (*v. infra*) is needed to arrive at even a provisional relationship between the direct and indirect heats. The data were therefore supplemented by a set of observations.

(II) At *p*H *c.* 9·5 (by Adair, Barcroft, Herkel, Hill, Keys & Roughton).

At this *p*H, both reduced and oxyhaemoglobin are almost entirely in the oxy-labile ionized form, and the primary reaction $O_2 + Hb^- \rightarrow O_2Hb^-$, is not complicated by any secondary ionic changes. In this range, haemoglobin is relatively stable, but owing to the greater affinity of Hb for O_2 at alkaline reactions, the concentrations of O_2 in the gas phase could not be estimated with nearly as great percentage accuracy as in (I)—hence the calculated values of the heat are subject to a much larger error. Had it been possible to work at *p*H 5·6, where reduced and oxyhaemoglobin are both almost entirely in the oxy-labile unionized form, this difficulty would have been obviated; unfortunately haemoglobin changes too readily to methaemoglobin at this acid reaction, for this to be feasible.

(III) The last section of this paper (by Roughton), deals with two theoretical problems, which have arisen from consideration of the data obtained under (I) and (II), viz.:

(*a*) The question of the interpretation of the Van't Hoff Isochore from the standpoint of Adair's [1925] intermediate compound hypothesis of the reaction between oxygen and haemoglobin, according to which the reaction proceeds in four stages.

(*b*) The theoretical basis of the relationship between the direct and indirect heats, in the *p*H range 6·0–9·0 when foreign buffers are present.

I. EXPERIMENTS AT *p*H 6·8.

BY S. GOLDSCHMIDT,[1] G. B. RAY AND F. J. W. ROUGHTON.

EXPERIMENTAL METHODS.

A. *The dissociation curves.*

(*a*) *Purification.* The haemoglobin from ox blood was purified by the method of Adair *et al.* [1921]. It was dialysed from 4 to 6 days at 0–1° against a *M*/15 phosphate buffer solution of *p*H 6·90 at 0°. Latterly the dialysis has been speeded up by rocking the dialysing sacs in a large shaker. The final solutions had an oxygen capacity of 20–26 volumes %: and were stored at 0·5° until required.

(*b*) Tests were made for the presence and amount of the so-called "inactive haemoglobin". A preliminary comparison of the amount of haemoglobin determined by the oxygen capacity with that obtained by the method of Stadie [1920] and the refractometer [Stoddard & Adair, 1923] gave a measure of the amount of haemoglobin which did not combine with oxygen. In none of the solutions used did this value exceed 5 %, nor did it increase appreciably during the period of experimentation.

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[1] From the University of Pennsylvania.

(*c*) *Equilibration* of the haemoglobin solutions with varying oxygen tensions was carried out in tonometers, similar in design to those described by Austin *et al.* [1922], with the solutions stored in a small accessory tube prior to equilibration. The gas mixtures were made according to the method of Krogh & Leitch [1919], namely, by washing the tonometer and the attached blood gas reservoir three times with oxygen-free nitrogen and, after a final exhaustion, admitting an amount of CO_2-free air sufficient to give the desired oxygen tension. A measured amount of fully or partially oxygenated haemoglobin was drawn into the reservoir and then nitrogen admitted to a known pressure, usually atmospheric.

(*d*) *The temperature.* Five different temperatures over the range 0–40° were used, the temperature being regulated to within $\pm 0{\cdot}1^\circ$. 10 min. were allowed for equilibrium to be attained.

(*e*) *Analysis of solutions.* The oxygen content of the solutions was, in most cases, determined by the Van Slyke manometric apparatus. All results were obtained in duplicate and checked to within 0·2 ml. O_2 per 100 ml., i.e. to within about 1 % oxyhaemoglobin. In one series of experiments the Barcroft differential manometer was used. In this case the oxygen unsaturation (or percentage reduced haemoglobin) was determined by finding out how much O_2 each sample took up on shaking with air. The oxygen capacity of the haemoglobin solution was determined, by the Van Slyke method, upon a mixed solution remaining from the equilibrated samples, this providing a check on any ill-effect resulting from manipulation. In no case was the exposure to low oxygen tensions detrimental to the haemoglobin, except in one experiment the results of which were discarded.

All the solutions were free from CO_2. This was of importance, since CO_2 is now known to have a direct effect on the oxyhaemoglobin dissociation curve apart from that due to its effect on the *p*H of the solution [Margaria & Green, 1933].

From the difference between the original and final contents of oxygen in the haemoglobin solution, and the data from the preparation of the gas mixtures, the final tension of oxygen at bath temperature was computed by the method suggested by Austin *et al.* [1922]. The final results of the blood analyses were corrected for dissolved oxygen from data given by Ferry & Green [1929] corrected for our experimental temperatures.

In the series of experiments in which the oxygen content of the solution was determined by the Barcroft differential manometer, the final oxygen in the gas phase was also determined by analysis in the Haldane apparatus.

B. *The measurement of the heat of the reaction.*

Most of the details of the experimental technique and procedure will be found in the earlier paper by Roughton [1935]. Here it need only be noted that in the heat measurements:

(*a*) The haemoglobin solution, which had been prepared for the dissociation curve determinations, was diluted with an equal volume of distilled water, so that the heat measurements should not impose an undue drain upon the supply of purified haemoglobin (of which in each set of experiments about 400 ml. were available). It is unlikely that this degree of dilution would have affected the heat per mol. of O_2 combining with Hb.

(*b*) In one of the thermos flasks were placed about 200 ml. of the completely reduced Hb solution, in the other an equal volume of 1 % boric acid solution. Since, owing to the shortage of haemoglobin, it was impossible to have the two solutions in the two thermos flasks[1] identical, the temperature drifts at the beginning and end of each experiment were more serious than usual; indeed in one or two cases the correction for drift, as applied in the standard manner, amounted to as much as 30 % of the total heat change. Such large drifts are no doubt also in part due to lack of sterility of the Hb solutions, and possibly to other slow secondary changes (such as denaturation) to which purified Hb (especially at a pH relatively so acid as 6·8) may be more prone than is whole blood. In experiments, particularly with haemoglobin several days old, there was reason to suspect such changes. It may be remembered that Brown & Hill [1923] noted that whole blood was by far the most satisfactory material to work on. Had it been possible to have the same Hb solution in each thermos flask, such changes might have cancelled each other out.

It would certainly have been more satisfactory if the drifts had not been so large, but their largeness does not shake our faith in the degree of agreement found below between the heat as obtained (*a*) directly and (*b*) indirectly from the dissociation curves, for in correcting for the drifts, the observer has no inkling of what the final result will be until the very end of the calculation.

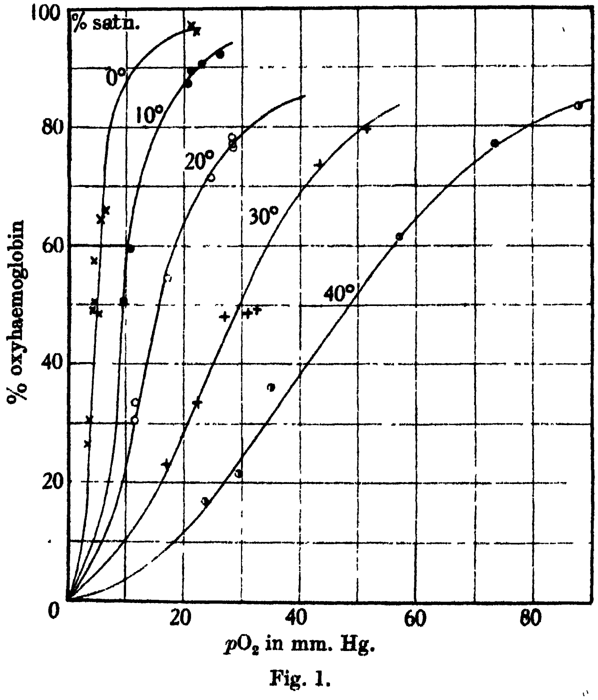

Fig. 1.

EXPERIMENTAL RESULTS AND CALCULATIONS.

A. *The effect of temperature on the dissociation curves.*

Experimental results. Fig. 1 demonstrates the effect of temperature on the dissociation curve of purified haemoglobin of the ox in $M/15$ phosphate buffer,

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[1] Unfortunately, owing to an accident at a critical moment, it was necessary to carry on with two rather ill-matched thermos flasks.

pH 6·90 at 0°. This family of curves represents the influence of temperature on the shape of the dissociation curve. It is noteworthy that the curves are sigmoidal at all temperatures. A discussion of some previously existing data on this subject may be found in Chapter XVI of Barcroft's book [1928].

The points upon the curves were obtained from two separately prepared solutions of haemoglobin from different ox bloods, yet the curves drawn from one set of determined points serve equally well for the other.

Inspection of the curves confirms the view of previous writers that the effect of temperature is to alter the scale rather than the shape of the dissociation curve. That this is quantitatively true can be shown by multiplying the abscissae of the various smoothed curves by appropriate coefficients, whereupon the curves all reduce to (within experimental errors) a common dissociation curve which is very close to that obtained by Forbes & Roughton [1931] for dilute sheep's blood.

This result is demonstrated best, not by a graph but by Table I.

Table I.

% O_2Hb	0° $pO_2 \times 6$	10° $pO_2 \times 3{\cdot}4$	20° $pO_2 \times 1{\cdot}9$	30° $pO_2 \times 1{\cdot}02$	40° $pO_2 \times 0{\cdot}6$	Forbes-Roughton $pO_2 \times 10{\cdot}4$
20	—	—	17·8	16·5	17·2	16·2
30	22·0	—	21·5	21·2	21·8	22·5
40	27·0	—	25·9	25·7	26·5	27·0
50	30·0	31·8	29·2	30·1	31·0	31·2
60	33·6	35·0	34·8	35·0	35·8	35·8
70	—	—	43·7	41·7	41·8	41·6
80	—	—	57·0*	52·0	50·6	50·0

The column of figures on the right of Table I are calculated from the curve given in their Fig. 5 by Forbes & Roughton. With the exception of one point asterisked, the effect of temperature at the different parts of the dissociation curve is uniform enough. From the multipliers used in Table I the temperature coefficient per 10° (Q_{10}) of the curves can be obtained. From 0–10° $Q_{10} = 1{\cdot}76$, 10–20° $Q_{10} = 1{\cdot}79$, 20–30° $Q_{10} = 1{\cdot}86$, 30–40° $Q_{10} = 1{\cdot}60$. These figures do not indicate any marked "breaks" in the effect of temperature, although to exclude this possibility beyond doubt, a much greater number of temperatures would be necessary.

Relation between log [O_2 concentration] and 1/T at equilibrium. Fig. 2 shows the relation between log $pO_2 \times 273/T$ at equilibrium and $1/T$ for (1) 30 % O_2Hb, (2) 50 % O_2Hb and (3) 70 % O_2Hb. $pO_2 . 273/T$ is the pressure which the oxygen would have exerted at the standard temperature of 0° in each case, and hence is proportional to the concentration of O_2 in the gas phase whatever the value of T. The curves obtained are practically linear and of very nearly the same slope in each case, thus showing that the effect of temperature on the O_2-Hb equilibrium, as judged by this criterion also, is devoid of the "breaks", referred to as possible in the introduction, and furthermore is roughly the same at different parts of the dissociation curve, as has been found also to be the case by Barcroft [1928] for the rougher data given in Barcroft & King's curves [1909].

Comparison of Q as measured directly with value of Q_v' as calculated indirectly on assumption that the Van't Hoff Isochore can be applied. From Fig. 2 the calculated value of Q_v' using

50% oxyhaemoglobin	= 9400 calories
70% oxyhaemoglobin	= 9650 calories
30% oxyhaemoglobin	= 9300 calories
Mean	9450 calories

The heat so calculated is that of the combination of 1 g. mol. O_2 in the gaseous phase with haemoglobin in the liquid phase. Since the calculated value of the heat appears to be the same at these different degrees of saturation, the direct value should also be constant over this range, and comparison should be justifiable, therefore, with the heat measured calorimetrically when the haemoglobin is brought from 0 to 70 % saturation with oxygen.

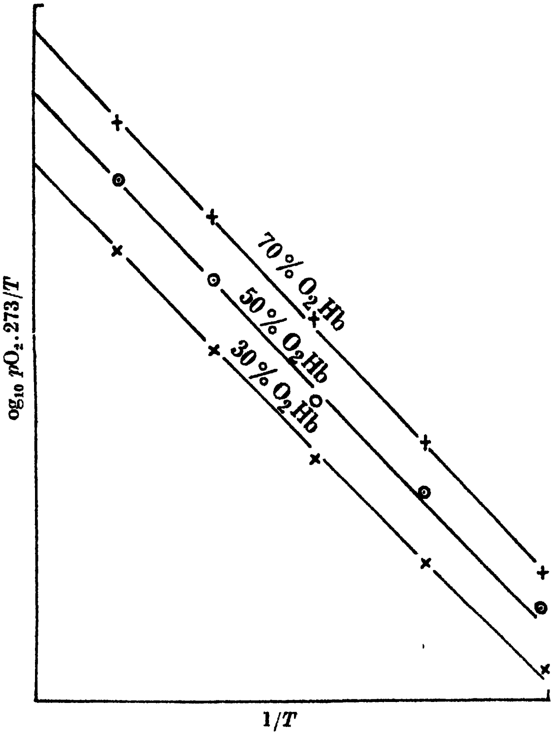

Fig. 2.

The directly measured value of Q for the same sample of haemoglobin was found to be 9000 ± c. 700 calories over the range 0 to c. 70 % oxyhaemoglobin. In a second comparison on haemoglobin, prepared from the blood of another ox:

The value calculated from the effect of five different temperatures (the same as in Fig. 2) on the dissociation curve = 9450 calories.

The directly measured heat at 19° = 9700 calories.

It may therefore be concluded that the value of Q, as measured directly for haemoglobin in $M/15$ phosphate buffer pH 6·8, agrees within experimental error with the value of Q_v', as calculated from the Van't Hoff Isochore in the form of equation (1).

Brown & Hill [1923] found, in the case of borated blood, an appreciable effect of temperature upon the value of Q (direct), viz. about −1200 calories per 10°. This they attribute, on Kirchoff's principle, to a slight difference in the specific heats of oxygenated and reduced haemoglobin solution.

No study has been made yet of the effect of temperature upon Q (direct) for the purified ox haemoglobin, pH 6·8, used in these experiments, but as regards

Q_v' (as calculated) the fact that log $pO_2 . 273/T$ plotted against $1/T$ gives a straight line over the range 0–40° suggests that the indirect value, Q_v', is not affected by temperature over this range, though owing to the rather considerable effect of experimental error in the dissociation curves on the calculation of Q_v' it would be unwise to exclude the possibility altogether.

II. EXPERIMENTS AT *p*H 9·5.

By G. S. ADAIR, J. BARCROFT, W. HERKEL, R. M. HILL,
A. B. KEYS AND F. J. W. ROUGHTON.

EXPERIMENTAL METHODS.

(*a*) *Preparation of the haemoglobin solutions.* The haemoglobin solutions were prepared from ox blood, by a procedure similar to the method (No. 1) for the preparation of carbon monoxide haemoglobin described by Adair & Adair [1934]: instead of adjusting the *p*H values by dialysis against a standard buffer, the haemoglobin was dialysed against distilled water and the solution was made alkaline with *N* sodium hydroxide, which was added slowly, with continuous stirring, in order to minimize the risk of denaturation, until the final concentration of sodium hydroxide was *N*/16. The titration curves of Hastings *et al.* [1924] indicate that the *p*H value of such solutions exceeds 9·5, which is sufficient to ensure that both oxygenated and reduced haemoglobin are present almost entirely in the oxy-labile ionized form.

Two separate preparations were made in this way:

(i) contained a total haemoglobin content (determined refractometrically) of 0·00996 equivalent/litre.[1] The oxygen capacity determined gasometrically was 0·00899 equivalent/litre, so that 10 % of the haemoglobin was "inactive",

(ii) contained a total haemoglobin content of 0·0102 equivalent/litre, of which 0·00978 equivalent/litre were gasometrically active. The "inactivation" in this case thus only amounted to 2·4 %.

(*b*) *Equilibration and analysis of the Hb solutions.* The solutions were equilibrated in the tonometers described by Barcroft [1934] in a constant temperature bath:

(i) The oxygen contents of the equilibrated solutions were determined in duplicate by means of the Van Slyke manometric apparatus. The duplicates, on the average, checked to within 0·2 ml. O_2 per 100 ml. solution, i.e. to within about 1 % oxyhaemoglobin.

(ii) The oxygen pressure in the gas phase after equilibration was determined, in the usual way, by means of the Haldane gas analysis apparatus. In some cases it was also calculated from a knowledge of (α) the total oxygen introduced into the tonometer at the start (i.e. the sum of the oxygen introduced into the gas phase and the oxygen introduced via the haemoglobin solution), (β) the oxygen content of the haemoglobin solution at the end of equilibration and (γ) the volumes of the gas phase and liquid phase in the tonometer. The oxygen pressure, so calculated, agreed to within 0·1 mm. Hg on the average with the pressure as determined by the direct analysis. The degree of agreement is well within the limits of experimental error of the methods.

(iii) The temperature. Experiments were done over the temperature range 9–39°. No observations were made at the lower temperature of 0°, as in the work at *p*H 6·8, since in the present case the oxygen pressures at 0° would have only been of the order of 0·5 mm. Hg, and therefore too small for accurate analysis by the ordinary Haldane apparatus.

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[1] 1 equivalent of haemoglobin = the weight containing 56 g. iron = 16,800 g.

(c) *The heat of the reaction.* The details of the measurement were almost the same as at pH 6·8, but since larger volumes of haemoglobin solution were available and much less was required for the dissociation curve determinations, sufficient haemoglobin solution was left over for both the thermos flasks to be filled with the same solution. This, as pointed out in Section I, gives increased accuracy over that obtainable with differing solutions in the two flasks: furthermore, in these experiments the latter were well-matched in size and thermal conductivity. Greater confidence can hence be placed in the direct calorimetric measurements of this Section than in those of Section I.

EXPERIMENTAL RESULTS AND CONCLUSIONS.

Two different preparations of purified ox haemoglobin solution were used.

Fig. 3 shows the dissociation curve points obtained on the first preparation at temperatures 9, 19, 29 and 39°. From these data, the average value[1] of the heat of the reaction O_2 gas (at constant volume) + haemoglobin $\rightleftharpoons$ oxyhaemoglobin was calculated for the three temperature ranges, viz.

9–19° heat = 12,500 calories,
19–29° heat = 12,400 calories,
29–39° heat = 13,700 calories.

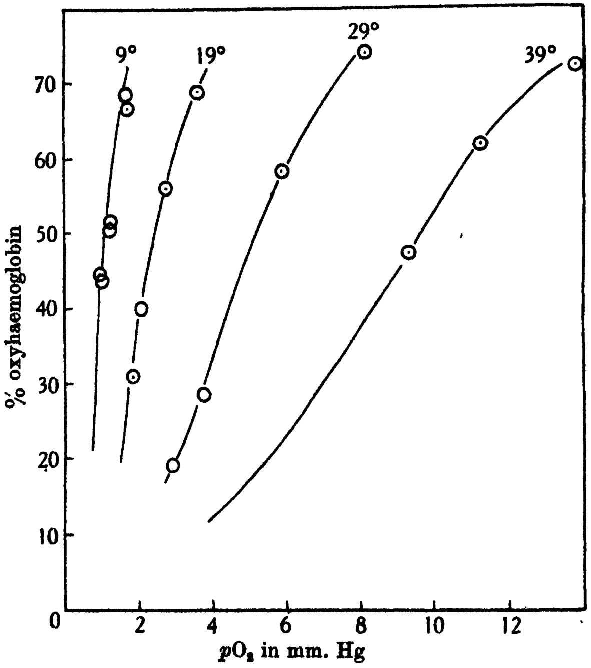

Fig. 3.

The heat, as measured directly at 19°, was 12,400 calories. This is to be compared with the mean of the calculated values for the ranges 9–19° and 19–29°, viz. 12,450 ± 1200 calories.

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[1] By using, for the calculations, as much of the smoothed dissociation curve as was available at each temperature.

From similar data for the second haemoglobin solution, the calculated values for the heat are as follows:

9–19° heat = 15,100 calories
19–29° heat = 13,100 calories
Average = 14,100 calories ± *c.* 1000.

The direct measurement of the heat for this reaction gave 14,250 calories at a temperature of 19°. The indirect measurements may be subject to an error of 1000 calories, since, as already emphasized, they are based on measurements with relatively low gas pressure.

Within the limits of experimental error the calculated values of the heat agree with those determined directly by the calorimetric method at this *p*H also.

THEORETICAL DISCUSSION.

By F. J. W. ROUGHTON.

THEORETICAL CONSIDERATION OF THE RELATION BETWEEN THE HEAT OF THE REACTION AND THE EFFECT OF TEMPERATURE ON THE DISSOCIATION CURVE.

The simple form of the Van't Hoff Isochore used so far in this work, viz.:

$$Q_v' = R \times \frac{T_1 T_2}{T_1 - T_2} \times \log_e \frac{C_2}{C_1}.$$

can be shown to be a necessary consequence both of:

(*a*) Hufner's theory, according to which the reaction, from the mass action point of view proceeds simply as

$$1 \text{ mol. Hb} + 1 \text{ mol. } O_2 \rightleftharpoons 1 \text{ mol. } O_2 \text{ Hb}.$$

(*b*) Hill's theory, i.e. that the reaction proceeds in the form

$$Hb_n + nO_2 \rightleftharpoons (HbO_2)_n.$$

Neither of these earlier theories, however, can now be regarded as valid for mammalian haemoglobin: their place has at present been taken by the intermediate compound hypothesis of Adair [1925]. How does the simple form of the isochore fare on this theory?

Writing the reduced haemoglobin molecule as Hb_4 to express the fact that it can combine reversibly with 4 molecules of oxygen, Adair's hypothesis states that the reaction occurs in four stages.

$Hb_4 + O_2 \rightleftharpoons Hb_4O_2$ equilibrium constant K_1, heat of reaction $= Q_1$ ......(2·1),
$Hb_4O_2 + O_2 \rightleftharpoons Hb_4O_4$ equilibrium constant K_2, heat of reaction $= Q_2$ ......(2·2),
$Hb_4O_4 + O_2 \rightleftharpoons Hb_4O_6$ equilibrium constant K_3, heat of reaction $= Q_3$ ......(2·3),
$Hb_4O_6 + O_2 \rightleftharpoons Hb_4O_8$ equilibrium constant K_4, heat of reaction $= Q_4$ ......(2·4).

This theory leads to the equilibrium equation

$$y = \frac{\%\text{ oxyhaemoglobin}}{100} = \frac{K_1C + 2K_1K_2C^2 + 3K_1K_2K_3C^3 + 4K_1K_2K_3K_4C^4}{4(1 + K_1C + K_1K_2C^2 + K_1K_2K_3C^3 + K_1K_2K_3K_4C^4)},$$

where C = concentration of oxygen in the gas phase.

These equations obviously do not, in general, lead to so simple a form of the Van't Hoff Isochore as that given in equation (1). First then we must ask whether there are any special conditions in which the simple equation (1) would be theoretically expected to be valid.

One of the basal assumptions made in the thermodynamic deduction of the Van't Hoff Isochore readily gives the clue. For equation (1) to be rigorously valid, the concentration of every reactant, except O_2, must be the same in the equilibrium at temperature T_1 as at T_2. This means that

(*a*) At any given percentage saturation y, the concentration of each intermediate compound must be unaltered by change of temperature. This can only be the case if

(*b*) The heats of the intermediate reactions, i.e. Q_1, Q_2, Q_3, Q_4, are all exactly equal.

The following conditions also follow from (*a*) and (*b*).

(*c*) The dissociation curve at T_2 must be an exact replica of the dissociation curve at T_1, i.e. it must be possible to make the two curves coincide exactly, just by multiplying the abscissae of one of them by an appropriate constant factor.

(*d*) The value of Q_y' as calculated from equation (1) must be the same, whatever value of y is chosen.

(*e*) Q_y' must be equal to Q, the heat as given by direct calorimetry, whatever the range of percentage saturation chosen in the calorimetric measurements.

Conversely if conditions (*c*), (*d*), (*e*) hold good, so then must also conditions (*a*) and (*b*).

In point of fact conditions (*a*) and (*b*) were implicitly assumed to be true by Adair [1925] in his derivation of the simple form of the isochore (equation (1)) on the intermediate compound hypothesis. These assumptions have not, however, been tested experimentally, there being at present no way of measuring either the concentrations of the individual intermediates or of the heats of their reactions with O_2, i.e. Q_1, Q_2, Q_3, Q_4. But the experimental work, both of previous investigators [summarized by Barcroft, 1928] and of these papers, shows that conditions (*c*), (*d*), (*e*) are, within experimental error, realized. Does it follow from this that, if the intermediate compounds exist, their heats of reaction with O_2 must all be equal?

This question has been tackled by numerical trial and error, with negative result, for it has been found that conditions (*c*), (*d*) and (*e*) can hold good (within experimental error) even when values of Q_1, Q_2, Q_3 and Q_4, which are widely different from one another are chosen. A single example will be sufficient to quote:

Let us, at temperature T_1, take the first set of values of K_1, K_2, K_3, K_4 put forward by Forbes & Roughton [1931] in Table VI of their paper, viz. $K_1=0{\cdot}4$, $K_2=0{\cdot}15$, $K_3=0{\cdot}06$, $K_4=4{\cdot}5$. These values fit Forbes & Roughton's dissociation curves within experimental error, and if the scale of O_2 pressures be suitably adjusted, they also fit the curves given in Section I.

Now suppose $Q_1=12{,}000$ calories, $Q_2=12{,}000$ calories, $Q_3=24{,}000$ calories, $Q_4=0$ calories. Choose a temperature T_2 so that $(K_1 \text{ at } T_2)/(K_1 \text{ at } T_1)=2$, therefore $(K_1 \text{ at } T_2)=0{\cdot}2$.

Then $(K_2 \text{ at } T_1)/(K_2 \text{ at } T_2)=2$, therefore K_2 at $T_2=0{\cdot}075$,
$(K_3 \text{ at } T_1)/(K_3 \text{ at } T_2)=4$, therefore K_3 at $T_2=0{\cdot}015$,
$(K_4 \text{ at } T_1)/(K_4 \text{ at } T_2)=1$, therefore K_4 at $T_2=4{\cdot}5$.

For simplicity we shall assume that the reaction is either so alkaline or so acid that there are no secondary effects due to changes in ionization of the haemoglobin when oxygenated.

Three representative points on the respective dissociation curves at temperatures T_1, T_2 have been calculated from these equilibrium constants and are given in Table II.

Table II.

Temperature T_1		Temperature T_2		Heat of reaction	
pO_2 mm. Hg	% saturation	pO_2 mm. Hg	% saturation	Indirect	Direct
1	10·07	2	9·98	12,090	12,120
2·5	36·30	5	35·95	12,150	12,150
5	77·69	10	77·74	11,990	12,110

It is obvious from the table that the two dissociation curves, if the pressure scale for the T_2 curve is compressed to one half, could not experimentally be separated from one another except by methods which were much more accurate than those at present available, i.e. condition (*c*) holds within experimental error.

The fifth column gives the heat as calculated from the effect of temperature on the oxygen pressure necessary to give equilibrium at the three different percentage saturations, shown in the second column of Table II, equation (1) being used for the calculation. The values again are too close to be separated by experiment, i.e. condition (*d*) holds within experimental error.

Finally column 6 gives the values which would be obtained by direct measurement of the heat per g. mol. O_2 combining when the haemoglobin is brought from zero percentage saturation to each of three percentage saturations shown. The mode of calculation of these values may be illustrated by an example:

At temperature T_1, pO_2 1 mm., equations (2·1), (2·2), (2·3), (2·4) give:

$$\begin{aligned}[Hb_4] &= 67{\cdot}60\% \text{ of total haemoglobin}\\ [Hb_4O_2] &= 27{\cdot}04 \quad ,,\\ [Hb_4O_4] &= 4{\cdot}04 \quad ,,\\ [Hb_4O_6] &= 0{\cdot}24 \quad ,,\\ [Hb_4O_8] &= 1{\cdot}08 \quad ,,\end{aligned}$$

Therefore heat per g. mol. O_2 combining, if directly measured over the range 0–10·07 % O_2Hb would be

$$=\frac{\left\{\begin{array}{l}(27{\cdot}04\times 12{,}000)+4{\cdot}04\times(12{,}000+12{,}000)\\ \quad +0{\cdot}24\times(12{,}000+12{,}000+24{,}000)\\ \quad +1{\cdot}08\times(12{,}000+12{,}000+24{,}000+0)\end{array}\right\}}{(27{\cdot}04)+(2\times 4{\cdot}04)+(3\times 0{\cdot}24)+(4\times 1{\cdot}08)}=12{,}120 \text{ calories.}$$

Column 6 shows that the directly measured heats would also be practically independent of the percentage saturation, and would be within experimental error, though not mathematically equal to the indirectly calculated heats. So condition (*e*) would also hold good.

Thus, in spite of the large differences between the heats of the several intermediate reactions, the haemoglobin would behave over the range 10–80% saturation practically in the same way as it would do if the heats of the intermediate reactions were all the same. The range mentioned represents the limits usually chosen in such studies, since above 80% the dissociation curve becomes too flat for accurate calculations to be made, whereas in the region below 10% the degree of saturation and O_2 pressure are too small to be measured precisely enough. If, however, very accurate measurements could be made at the extreme top of the dissociation curve, i.e. between 99 and 100%, the discrepancies postulated in our example would be brought to light, for in this special range, the curve would depend practically on the value of K_4 alone, and would thus be unaffected by temperature. This region of the dissociation curve, the importance of which has been emphasized elsewhere [Roughton, 1934] has not yet been made accessible to accurate measurement of the type required. These arguments thus lead to the following rather disappointing conclusions:

(i) That constancy, within experimental error, of the heat of reaction of haemoglobin with oxygen, over the whole range of percentage saturation, whether measured directly or calculated indirectly, does not in itself tell us anything about the heats of the intermediate reactions, nor indeed does it either support or disprove their existence.

(ii) That the simple form of the Van't Hoff Isochore (*v.* equation (1)), though a necessary consequence of the earlier theories of Hufner and of Hill, is not a necessary consequence of the intermediate compound theory, though it may, on the latter theory, be approximately or even exactly correct.

THE RELATION BETWEEN THE DIRECTLY MEASURED HEAT AND THE INDIRECTLY CALCULATED HEAT WHEN FOREIGN BUFFERS ARE PRESENT.

The equation $\log_e \frac{C_1}{C_2} = Q_v' (1/T_1 - 1/T_2)$ would not be expected thermodynamically to yield a value of Q_v' agreeing exactly with the directly measured value of Q if the *p*H of the haemoglobin solution is such that there is a change of *p*H on oxygenation. For, in such a case, the concentrations of the oxy-labile ionized and oxy-labile unionized fractions at T_1 and T_2 may not be the same, even if the total concentrations of oxyhaemoglobin and reduced haemoglobin at the two temperatures T_1 and T_2 are the same. The difficulty will only be avoided if the reaction is either so acid that the haemoglobin (oxy- and reduced) is all in the oxy-labile unionized form, or so alkaline that the haemoglobin is all in the oxy-labile ionized form. Of these, only the latter is at present accessible to experimental observation, and it was for the sake of simplifying the issue in this manner that the studies recorded in Section II were made.

We have previously shown [Roughton, 1935] how to calculate, in the intermediate range of *p*H, the relation between the directly measurable heat of reaction and the *p*H and buffer content of the solution; reference should be made to this treatment for a full understanding of the symbols used below. We shall now do the same as regards the indirectly calculable heat. In order to handle the problem, it is necessary to make certain assumptions, which, though not yet experimentally verified, are reasonable ones and, if accepted, lead to the conclusion that Q_v' and Q should, in fact, agree with one another within experimental error even though such an exact equality is not thermodynamically inevitable.

The principal assumption to be made concerns the relation between the oxygen concentration, C, required to produce a given degree of saturation, and the hydrogen ion concentration of the solution. The simplest assumption appears to be of the type

$$C = C_a \frac{\mathrm{H} + K_R}{\mathrm{H} + K_O} \times \frac{K_O}{K_R} \qquad \text{......(3),}$$

where C_a = concentration required when the reaction is so alkaline that the haemoglobin is completely ionized as regards the oxy-labile portion; K_O, K_R are the oxy-labile ionization constants of oxy- and reduced haemo-globin respectively.

Table III. *Relation between oxygen pressure for 50 °/₀ saturation and hydrogen ion concentration.*

(Data of Ferry & Green [1929] for horse haemoglobin at 25°.)

$$\text{If } C = C_a \frac{\mathrm{H} + K_R}{\mathrm{H} + K_O} \times \frac{K_O}{K_R}.$$

*p*H	6·77	7·0	7·38	7·65	7·95	8·4	8·8
H	1.7×10^{-7}	1.0×10^{-7}	4.17×10^{-8}	2.24×10^{-8}	1.18×10^{-8}	4.0×10^{-9}	1.32×10^{-9}
C* (observed)	9·7	8·0	4·8	3·6	2·0	1·3	1·1
C* calculated (i)	10·0	7·5	4·4	2·9	2·0	1·4	1·1
(ii)	10·5	8·2	5·5	3·5	2·2	1·4	1·1

* C = concentration of oxygen in gas phase, expressed as $\left(\frac{\text{g. mol.}}{\text{litre}}\right) \times 1000$.

(i) $K_O = 2 \times 10^{-7}$, $K_R = 9.4 \times 10^{-9}$.
(ii) $K_O = 2 \times 10^{-7}$, $K_R = 6.8 \times 10^{-9}$.

If $K_O = 2\times10^{-7}$, and K_R lies between 7×10^{-9} and 9×10^{-9}, this equation gives a reasonably good fit for the relation between C and H for 50 % saturation of the haemoglobin (horse) in the case of Ferry & Green's data [1929], as shown in Table III.

If equations (1) and (3) are valid at different temperatures

$$\frac{-Q_v'}{RT^2} = +\frac{\partial \log_e C}{\partial T} = \frac{\partial \log_e C_a}{\partial T} + \frac{\partial \log_e (\mathrm{H}+K_R)}{\partial T} - \frac{\partial \log_e (\mathrm{H}+K_O)}{\partial T} + \frac{\partial \log_e K_O}{\partial T} - \frac{\partial \log_e K_R}{\partial T} \quad ......(4).$$

If the respective variations of C_a, K_O, K_R and H with temperature are known, then the value of $\frac{\partial \log_e C}{\partial T}$ is calculable, and hence the indirect value for the heat of reaction. Of these

$$\text{(i)} \quad \frac{\partial \log_e C_a}{\partial T} = \frac{-Q_{Hb^-}}{RT^2},$$

where Q_{Hb^-} = heat of the reaction $O_2 + Hb^- \rightarrow O_2Hb^-$ = 13,400 calories [Roughton, 1935].

$$\text{(ii)} \quad \frac{\partial \log_e K_O}{\partial T} = \frac{Q_O}{RT^2},$$

where Q_O = heat of oxy-labile ionization of oxyhaemoglobin which is taken = 9000 calories.

$$\text{(iii)} \quad \frac{\partial \log_e K_R}{\partial T} = \frac{-Q_R}{RT^2},$$

where Q_R = heat of oxy-labile ionization of reduced haemoglobin.

With the aid of (i), (ii) and (iii) equation (4) thus becomes

$$\frac{-Q_v'}{RT^2} = \frac{-Q_{Hb^-}}{RT^2} - \frac{Q_O}{RT^2} + \frac{Q_R}{RT^2} + \frac{\partial \log_e (\mathrm{H}+K_R)}{\partial T} - \frac{\partial \log_e (\mathrm{H}+K_O)}{\partial T}.$$

Let Q_{Hb} = the heat of the reaction $O_2 + Hb \rightarrow O_2Hb$. Then by the Conservation of Energy

$$Q_O + Q_{Hb^-} = Q_R + Q_{Hb} \quad ......(5).$$

Therefore
$$-\frac{Q_v'}{RT^2} = \frac{Q_{Hb}}{RT^2} + \frac{\partial \log_e (\mathrm{H}+K_R)}{\partial T} - \frac{\partial \log_e (\mathrm{H}+K_O)}{\partial T},$$

or
$$Q_v' = Q_{Hb} - RT^2\frac{\partial \log_e (\mathrm{H}+K_R)}{\partial T} + RT^2\frac{\partial \log_e (\mathrm{H}+K_O)}{\partial T} \quad ......(6).$$

According to Roughton [1935], Q_{Hb} = 11,400 calories, and therefore $Q_{Hb^-} - Q_{Hb}$ = 2000 calories, so $Q_R - Q_O$ must, by equation (5), also be taken as 2000 calories, i.e. the heat of combination of oxy-labile H ions with reduced haemoglobin should be appreciably greater than with oxyhaemoglobin. It would be interesting but difficult to test this by direct calorimetric experiments.

According to Roughton [1935], the corresponding value of the directly measurable heat is given by the equation

$$Q = Q_{Hb^-}\frac{K_R}{K_R+\mathrm{H}'} + Q_{Hb}\frac{\mathrm{H}'}{K_R+\mathrm{H}'} + (Q_A - Q_H)\left(\frac{KA}{\mathrm{H}'+K} - \frac{KA}{\mathrm{H}+K}\right) \quad ......(7).$$

Where H, H′ = the hydrogen ion concentrations of the solution after and before oxygenation respectively; A = molar concentration of the foreign buffer/Hb concentration in equivalents/litre; K = ionization constant of the foreign buffer, Q_A its heat of ionization; Q_H = heat of ionization of haemoglobin.

Equation (6) is of quite different form from equation (7) and hence the value of Q_v' calculated therefrom should not necessarily turn out to be the same as the value of Q calculated from equation (7).

(iv) The variation of [H] with temperature.

If the [H] of a haemoglobin solution at temperature T_1 and a certain percentage saturation is known, the [H] of the same solution (at the same percentage saturation) can be calculated at temperature T_2 by means of an equation based on the following consideration.

When the temperature is changed the titration curve, both of oxyhaemoglobin and reduced haemoglobin, is shifted parallel to itself by 0·24 pH according to Stadie & Martin [1924]. Hydrogen ions are thus released from combination with haemoglobin by rise of temperature and are taken up by the foreign buffer.

If, for example, $T_2 - T_1 = 10°$,

pH_1, pH_2 are the respective pH values at T_1, T_2,
H_1, H_2 are the respective hydrogen ion concentrations at T_1, T_2.

Then $\beta\,[0{\cdot}24-(pH_1-pH_2)]$ = hydrogen ions ionized from haemoglobin owing to the change of temperature, where β = total buffer power of the haemoglobin solution.

These must be practically all taken up by the foreign buffer. If K_1, K_2 are the respective ionization constants of the foreign buffer acid at temperatures T_1, T_2, then the amount taken up by the foreign buffer

$$=A\left[\frac{K_1}{K_1+H_1}-\frac{K_2}{K_2+H_2}\right].$$

Therefore $$\beta\,[0{\cdot}24-(pH_1-pH_2)]=A\left[\frac{K_1}{K_1+H_1}-\frac{K_2}{K_2+H_2}\right] \qquad \ldots\ldots(8).$$

The change in H with temperature can thus be calculated, equation (8) being rapidly and simple soluble, by the method of successive numerical approximation. All requirements for the calculation of the "indirect heat" are now to hand. Such calculations have been carried out for haemoglobin of concentration 0·01 equivalent/litre in

(i) $M/15$ phosphate pH 6·695, $\beta = 2{\cdot}27$;
(ii) $M/15$ phosphate pH 7·042, $\beta = 2{\cdot}27$.

The percentage saturation chosen for the calculation of Q was 50 %, the temperature range 8–18°, and the following values were assumed for the ionization constants:

pK_{O} at 18° = 7·00, at 8° = 7·24.
pK_{R} at 18° = 8·50, at 8° = 8·80.
pK (phosphate) at 18° = 6·80, at 8° = 6·83.

These assumptions conform precisely with those adopted in Table III of the previous paper [Roughton, 1935] for (i) $pH = 6{\cdot}67$, $\beta_S = 2{\cdot}0$, (ii) $pH = 7{\cdot}0$, $\beta_S = 2{\cdot}01$, for the values of the ionization constants at 18° are the same as those used previously in the direct heat calculations and the values of the constants at 8° have been calculated from the corresponding values at 18° by means of the Van't Hoff Isochore, the values assumed for the various heats of ionization being those which were used in the direct heat calculations, viz. $Q_{\mathrm{O}} = 9000$ calories, $Q_{\mathrm{R}} = 11{,}000$ calories, Q (phosphate) = 1000 calories.

Table IV shows that the indirect heat and the direct heat, calculated in this manner, agree with one another empirically to within 150 calories—a figure which is well within the limits of error of either of these heats, when determined experimentally.

A similar degree of agreement in the calculations has also been found in the other cases dealt with in Table III of the previous paper [Roughton, 1935], viz. haemoglobin in $M/6$ borate buffer solution pH 7·6, and CO_2-free haemoglobin,

Table IV. *Comparison of direct and indirect heats for haemoglobin of concentration = 0·01 equivalent/litre in* M/15 *phosphate.*

*p*H	Direct heat Q range 0–100 % saturation	Indirect heat Q_{50}'
6·695	9930	10,000
7·042	9120	9,250

without added buffer, *p*H *c.* 8·4. Although a generalization would perhaps be unwise there seem fair grounds for concluding that, if the assumptions mentioned above (which, though reasonable, are not as yet experimentally verified) be correct, the heat of combination of oxygen with haemoglobin as measured directly, should agree with the heat, as calculated indirectly by means of the Van't Hoff Isochore, not only in (i) the theoretically simple regions, namely those of very acid or of very alkaline reaction but also in (ii) the relatively complicated situation, which arises when there is present in addition to the haemoglobin a foreign buffer with a heat of ionization very different from that of haemoglobin.

CONCLUSIONS.

The experimental data of this paper have shown that the effect of temperature upon the oxyhaemoglobin dissociation is related to the heat of the reaction by means of the simple form of the Van't Hoff Isochore given by equation (1), within the limits of experimental error. This statement holds good, not only for the theoretically simpler experiments at alkaline *p*H, but also in the more complex conditions which obtain at *p*H 6·8, in the presence of foreign buffer (*M*/15 phosphate buffer).

The significance of this agreement has been reconsidered in the light of the newer knowledge of the mechanism of the oxygen-haemoglobin reaction, which Adair's work [1925] on the molecular weight and Van Slyke's work [summarized by Peters and Van Slyke, 1931] on the titration curves of oxy- and reduced haemoglobin have given us. The theoretical treatment of this section—somewhat cumbersome it must be admitted—shows that the success of the simple form of the Van't Hoff Isochore (1) is compatible, within the limits of experimental error—though not necessarily with mathematical exactitude—with the intermediate compound hypothesis of Adair and with the concept of the oxy-labile ionization of haemoglobin, though it neither strengthens nor weakens the basis on which either of these views at present rests. Although the thermochemical study of the oxygen-haemoglobin reaction has thus brought forth new, and in some cases interesting, data it has not as yet shed any essentially fresh light on the nature of the reaction. This is disappointing in view of the hopes which earlier workers attached to this mode of approach.

The procedure for calculating the relation between the directly measured heat and the indirectly calculated heat when foreign buffers are present is of a general kind and could be applied to other cases, besides the special ones considered in the last subsection. An important case to consider, from the physiological point of view, would be that which exists in blood *in vivo*, wherein the red cell *p*H is 7·0–7·3 and there is much foreign buffer present, namely in the form of the HCO_3—H_2CO_3—CO_2 system: this has not yet been done, owing to lack of a further essential piece of knowledge, namely the heat of direct combination of CO_2 with haemoglobin in the carbamino-form. Recent work has shown that under physiological conditions the latter reaction is as intimately connected with the oxygenation of haemoglobin, as is the oxy-labile ionization reaction, to which so much attention has hitherto been paid above. The carbamino-reaction has been left out of account in the present paper, because in all the experiments here quoted, the solutions were freed of CO_2 and hence contained no appreciable amount of carbamino-compounds.

SUMMARY.

1. The effect of temperature on the oxyhaemoglobin dissociation curve has been studied on solutions of purified haemoglobin

(*a*) at *p*H 6·8, in $M/15$ phosphate buffer;
(*b*) at *p*H 9·5.

The results are essentially the same in the two cases, but for reasons given in the text, have been more fully and accurately worked out in (*a*).

2. Temperature alters the scale, but not the shape, of the dissociation curve: thus the value of the heat, Q_y', as calculated from the Van't Hoff Isochore

$$Q_y' = \frac{RT_1T_2}{T_1 - T_2} \log_e C_1/C_2,$$

is independent of y, the percentage saturation chosen for the calculation. The other symbols are defined in the text.

3. The value of Q_y' thus calculated agrees, within the limits of error, with the directly measured heat of the reaction, Q, both at *p*H 6·8 (average value 9350 calories) and at *p*H 9·5 (average value 13,300 calories).

4. The theoretical aspects of the comparison mentioned under 3 have not, hitherto, been fully considered in connexion with (i) the possible existence of compounds intermediate in composition between completely reduced haemoglobin, Hb_4, and fully oxygenated haemoglobin Hb_4O_8; (ii) the presence of haemoglobin both in the oxy-labile ionized form Hb^- and the oxy-labile unionized form Hb. One of the objects of this paper was to fill up these gaps.

5. It is pointed out that if intermediate compounds such as Hb_4O_2, Hb_4O_4, Hb_4O_6 be present, the overall heat of reaction measured directly need not necessarily agree with the value of Q_y' as obtained by calculation, nor need either heat be independent of the range of percentage saturation chosen, if the heats of the intermediate reactions vary. An example, worked out in the paper, shows, however, that the heats of the intermediate reaction can be quite different from one another without there ensuing any appreciable discrepancy between Q and Q_y', or any dependence of either upon the percentage saturation y. Experimental agreement between Q and Q_y' and independence of either of y does not therefore prove or disprove the existence of intermediate compounds.

6. The problem of relating the indirectly calculated heat Q_y' to the *p*H and foreign buffer content of the haemoglobin solution in the *p*H range 6·0–10·0 is also worked out. Numerical computations for certain typical cases of this kind show that empirically the value of Q_y' should agree closely with the directly measured value of Q even though such agreement is not thermodynamically necessary.

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CCXCVII. CONDUCTOMETRIC METHOD FOR THE MICRO-DETERMINATION OF UREA.

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In view of the great physiological importance of urea in protein metabolism, its diagnostic significance in diseases and its wide distribution in the tissues of organisms, numerous methods have been developed for its quantitative estimation in micro- and macro-quantities. Most of these methods involve its preliminary decomposition with urease and subsequent titrimetric or colorimetric estimation of the ammonia or the manometric determination of the CO_2 of the ammonium carbonate [Van Slyke, 1927]. A comprehensive survey of the methods available to the analyst is given by Peters & Van Slyke [1932].

Increasing importance is attached to micro-methods for elucidating physiological processes necessitating the study of reactions in single cells or in isolated regions of specific tissues. The micro-method developed by Linderstrøm-Lang [1933] for the estimation of ammonia represents an extreme refinement in technique, but in common with most other methods when applied to the determination of urea, involves a high degree of manipulative skill and is laborious and time-consuming. Colorimetric methods with an error of 2–3% are not sufficiently accurate; the manometric method with an accuracy of 1% and the micro-method of Conway [1933] are the two convenient methods available for the estimation of urea.

The applicability of conductometry to the study of the hydrolysis of urea, suggested by Bayliss [1925], was examined by Sastri & Sreenivasaya [1936] and it was shown that the progressive increase in electrical conductivity in the urea-urease system is a true measure of the hydrolysis of urea. It was further observed that the changes in conductance accompanying the hydrolysis were of considerable magnitude and could be employed when the quantity of urea was as low as 5×10^{-5} g. The present communication gives a critical study of the method and its application to a determination of urea in blood and other biological fluids.

EXPERIMENTAL.

The usual Kohlrausch bridge method was employed. A calibrated Kohlrausch slide wire—which can be read to 1 part in 100,000 when extension coils are in circuit—a 4-dial resistance box and an Arrhenius-Ostwald cell (cell constant 0·5879) formed the components of the circuit. With the cell employed about 5 ml. were required for immersing the electrodes. The cell was immersed in a thermostatic bath maintained at 30·0° ± 0·1°. The Audio-Oscillator (General Radio Co.) giving an alternating current of frequency 1000 was employed as the source of high frequency current and a telephone receiver was used for detecting the null-point. The capacitance of the cell was balanced by means of an air condenser connected in parallel with the resistance box.

2·5 ml. of the urea-containing solution were placed in the conductivity cell together with 1–3 ml. of *M*/20 phosphate buffer (*p*H 7·0) and sufficient conductivity water to make up the volume to 5·5 ml. and the contents of the cell well mixed. The cell was immersed in the thermostat, and after about half an hour the resistance was measured at 5-min. intervals till a constant value was obtained, indicating that the contents of the cell had reached the temperature of the thermostat. 0·5 ml. of a dialysed urease solution, raised to the temperature of the thermostat, was added and the cell contents well mixed. The addition of the enzyme was timed by a stopwatch; the first reading can be conveniently taken after an interval of 30 sec. Subsequent readings are taken at 1 min. intervals. The quantity of enzyme employed was such as to effect complete hydrolysis of the urea in 5–15 min. Table I gives the results obtained with 1·02 mg. and 0·10 mg. of urea.

Table I.

	1·02 mg. of urea		0·10 mg. of urea	
Time min.	Balancing resistance ohms	Bridge reading	Balancing resistance ohms	Bridge reading
	Before addition of enzyme.			
0	160	560	440	540
30	160	540	440	520
35	160	542	440	521
40	160	541	440	520
	After addition of enzyme.			
0·5	160	687	440	623
1	160	652	440	604
2	160	581	440	565
3	160	515	440	535
4	160	460	440	520
5	160	421	440	512
10	160	333	440	507
15	160	319	440	507
20	160	316	440	507
30	160	319	440	—

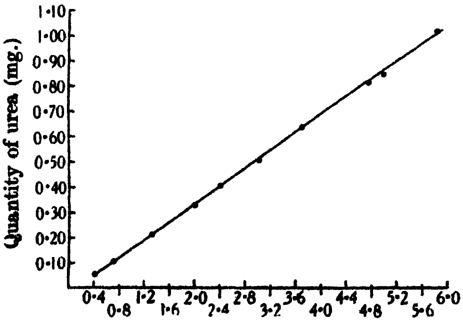

Fig. 1.

Table II. *Relation between change in conductivity and quantity of urea.*

Quantity of urea (mg.)	0·051	0·102	0·212	0·330	0·408	0·508	0·635	0·817	0·847	1·021
Change in conductivity × 10^4	0·42	0·72	1·34	2·00	2·43	3·03	3·72	4·75	4·98	5·60

The readings are plotted on a graph, time against resistance and by extrapolation the resistance at 0 time can be read. The total change in resistance gives a measure of the electrolyte released during the reaction and hence the quantity of urea.

The change in conductivity accompanying the hydrolysis of varying quantities of urea (0·05–1·0 mg.) was determined similarly, and from the readings obtained a graph (Fig. 1) showing the relation between quantity of urea and change in conductivity was prepared. Table II gives the readings obtained.

This graph could be used for obtaining urea values of different physiological fluids such as blood, urine and milk.

Applications. The method has been employed for the estimation of urea in blood, urine and milk. The soya enzyme used in these experiments was prepared by Van Slyke and Cullen's acetone precipitation method [1914]. To test the applicability of the method, two series of experiments were carried out: (1) known quantities of urea were added to a definite volume of blood, milk or urine and the quantity of urea thus added estimated by the conductometric method, and (2) the values obtained by the conductometric method were compared with those obtained by the well-known aeration-titration procedure of Van Slyke & Cullen [1916]. The results obtained with additions of known quantities of urea are tabulated in Table III.

Table III. *Estimation of added urea.*

	mg. urea estimated after addition to		
Quantity of urea added mg.	Blood (sheep) 0·20 ml.	Urine (human) 0·01 ml.	Milk (cow) 0·50 ml.
0·102	0·100	0·100	0·099
0·204	0·205	0·203	0·200
0·408	0·399	0·391	0·401

The difference in conductivities before and after addition of urea is a measure of the change due to the added urea. There is close agreement between the added and estimated values.

The results obtained by the conductometric and titrimetric methods are compared in Table IV. The quantities of blood and urine employed for the aeration-titration method are respectively 5 and 1 ml. The corresponding quantities for the conductometric method are 0·2 and 0·01 ml.

Table IV. *Comparison of aeration-titration and conductometric methods.*

	mg. of urea in			
		Urine (human) 1·0 ml.		
Method	Blood (sheep) 5·0 ml.	Sample 1	Sample 2	Sample 3
Aeration-titration	2·6	6·8	10·1	7·3
Conductometric	2·5	6·7	10·0	7·6

The close agreement between the two sets of values establishes the applicability of the conductometric method to the estimation of urea in physiological fluids.

The urea contents of some normal and pathological specimens of urine and blood (human), as well as those of milks, determined by the conductometric method are tabulated below.

Table V.

Material	Urea* mg. in 10 ml.
Human urine (normal)	75
„ (albuminous)	178
„ (jaundice)	82
„ (albuminous)	113
Human blood (normal)	3·1
„ (acute nephritis)	8·5
„ (mild nephritis)	5·8
Milk (cow)	2·2
„ (ass)	4·9
„ (ass, ultrafiltrate)	4·9

* The values for blood and urine approximate to those furnished by the Bowring Hospital, Bangalore, from whom the samples were obtained.

DISCUSSION.

The results given above show that conductometry furnishes an elegant means for the accurate estimation of urea in physiological fluids. In Table VI, an attempt has been made to compare the method with others in vogue with respect to minimum quantities of experimental material, rapidity and accuracy of estimation.

Table VI. *Estimation of urea in physiological fluids. A comparative study of methods.*

	1 Aeration-titration	2 Manometric	3 Colorimetric (ammonia distillation or Permutit absorption)	4 Hypobromite	5 Xanthydrol (Beattie, colorimetric)	6 Conductometric
Quantity of blood required (ml.)	3	0·2	0·5	0·5	<0·5	0·1
Accuracy (%)	1	1	2–3	Positive error >4	2–3	1
Time taken (min.)	Long time: involves isolation of ammonia	10	Long time: involves isolation of ammonia	3	Long time: involves filtration of ureide	5–10

It will be seen that the method, while it claims equality with the manometric procedure with respect to accuracy, possesses the merit of requiring smaller quantities of material. By choosing appropriate cells it should be possible to work with even smaller quantities. The molar concentration of ammonia in the cell, in our experiments, was as low as 1/1700; the cell contained 6 ml. of liquid and the distance between the electrodes was 1 cm.; by merely decreasing the distance between the electrodes, it will be possible to employ smaller quantities of fluid without greatly affecting the accuracy. The procedure is simple, does not require the use of standard solutions or many pieces of apparatus. There is hardly any need for determining the preformed ammonia by a duplicate experiment. The use of strong alkali carbonates for displacing the ammonia formed as a result of the hydrolysis of urea in physiological fluids involves the risk of the decomposition of associated amide-containing proteins with the liberation of ammonia therefrom. By reason of its simplicity and reliability, the conductometric method can be adopted by the trained medical practitioner for the routine examination of bloods and urines.

Conductometry can be employed not only for the estimation of urea but also for the determination of urease activity and in a similar manner for the estimation of arginine and arginase activity. For the study of the kinetics of urease and arginase, particularly when they are available only in micro-quantities, the method offers exceptional advantages: thus it permits of a number of readings in the initial stages of the action, not possible by chemical methods. The possibility of adopting this procedure for the determination of urea and arginine or their respective enzymes in tissues is now being examined.

SUMMARY.

A micro-method for the estimation of urea based on the measurement of the change of conductivity resulting from the hydrolysis of urea by urease has been described. The method gives reliable values for quantities as low as $0 \cdot 5 \times 10^{-5}$ g. of urea.

The method has been applied to the determination of urea in various physiological fluids and the values obtained compared with those obtained by macromethods.

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CCXCVIII. AMMONIA FORMATION IN IRRADIATED TISSUES.

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SINCE cells obtain their energy principally by the oxidation and splitting of sugars, the effect of radiation on their carbohydrate metabolism has been most widely investigated.

Holmes [1933], using cultures *in vitro* of embryonic kidneys, has studied the nitrogenous metabolism of irradiated cells by estimating the end-products urea and ammonia. She found that doses of γ-radiation over 14 hours did not produce an unequivocal effect on protein breakdown (as measured 48 hours after the treatment), but that carbohydrate breakdown was inhibited 40–50 % during the same period, showing the greater sensitivity of carbohydrate metabolism to radiation.

Lawrie & Robertson [1935] studied the effect of X-radiation on the growth and nitrogenous metabolism of a protozoon, and found definite effects, which, however, varied according to the duration of the exposure. It was shown earlier [Crabtree, 1935] that the glycolysis of tumour tissue can be almost completely checked by $\beta+\gamma$-radiation applied for 4 hours at low temperature, respiration remaining intact after similar treatment. It seemed desirable to discover if the suppression of glycolysis was accompanied by the liberation of ammonia, a reaction demonstrated by Warburg *et al.* [1924] as occurring in tumour and other normally glycolysing tissues when deprived of sugar.

The rate of this ammonia formation in the absence of sugar was shown to be roughly parallel with the rate of glycolysis characteristic of a wide range of tissues. That ammonia formation was linked to glycolysis and not respiration was confirmed by Salter & Robb [1934], who also showed that cyanide checked neither glycolysis nor ammonia sparing, but that iodoacetic acid checked both. Watchhorn & Holmes [1927] found that addition of glucose inhibited ammonia formation in cultures of tissues growing *in vitro*. They regarded this as an example of the protein sparing action of carbohydrate made evident *in vitro*. Since many workers have described the formation of lactic acid by cultures *in vitro* of tissues which, in the adult condition, exhibit no aerobic glycolysis, it is possible that the findings of Watchhorn & Holmes provide a further illustration of the relationship between ammonia formation and glycolysis.

Whereas the workers quoted above found ammonia formation to increase when glycolysis was checked by the absence of sugar, this same effect is produced when glycolysis has been suppressed by radiation and sugar is present. Deprivation of substrate or loss of the power to break down this substrate lead to the same end-result, an accumulation of ammonia. The origin of this ammonia is unknown. Warburg *et al.* [1924] suggested and Watchhorn & Holmes [1927] assumed that it was a product of protein breakdown, and that under normal physiological conditions sugar protects protein. Salter & Robb [1934] quote evidence suggesting that purine metabolism may be involved.

The increased ammonia formation accompanying the suppression of glycolytic function by irradiation at low temperature was at first thought to be only a further confirmation of the original finding of Warburg *et al.* [1924]. But further experiments in which irradiation was carried out under different conditions, and in which glycolysis remained unimpaired, showed that the increased ammonia formation observed was not necessarily coupled with glycolysis, but was a characteristic effect of the radiation itself. Irradiation at body temperature, aerobically, anaerobically or in the presence of cyanide, primarily damages the respiratory system whilst glycolysis proceeds normally. Yet under all these conditions radiation leads to a large increase in the rate of ammonia formation, showing that this phenomenon is independent of glycolysis. Under such conditions ammonia formation seems to be linked to respiration. In fact, if suitable normal tissues with low glycolysing powers are irradiated, the rate of ammonia formation increases in all cases, though to a variable extent. If it be assumed that increased ammonia formation implies increased protein degradation, then the generalization arising from this work (from the point of view of induced changes of metabolism) is, that radiation operates primarily by damaging carbohydrate metabolism, and this damage, whether to oxidative or splitting processes, leads to a secondary reaction in which protein is utilized more rapidly than in normal cells, or in isolated tissues preserved in artificial media for periods up to 5 or 6 hours. It is of interest to quote one of the earliest enquiries on the influence of X-rays on metabolic processes. Williams [1906] studied the action of X-radiation upon the excretion of uric acid in a clinical case of myelogenous leucaemia. He found that the amount of uric acid excreted during the period of irradiation was well above the normal limits, and moreover, that the total output of nitrogen exceeded the intake.

Experimental methods and results.

The apparatus used for irradiating tissue slices with $\beta+\gamma$-radiations has been previously described [Crabtree, 1935]. It was immersed in water at the appropriate temperature and the tissues irrigated with Ringer's solution containing glucose and bicarbonate (R.G.B.), either aerobically, anaerobically or containing $M/600$ HCN. The tumour tissue used was Jensen's rat sarcoma (J.R.S.), and rat liver, brain and testes were used as types of normal tissue, all possessing low aerobic glycolysis but varying in their power of glycolysing anaerobically. After irradiation, the slices were then transferred to small vessels containing 6 ml. of either R.G.B. or the same medium with glucose omitted, attached to manometers, and reincubated at 37·8° with shaking for 1 hour under aerobic conditions. The tissues were then dried and weighed, and ammonia was estimated in the 6 ml. of medium used. Control tissues were preserved, without irradiation, under similar conditions. At first, an initial measurement of the rate of ammonia formation was made to assess the effect of the experimental conditions on this process. This was discontinued when it was found that the difference between the initial rate of ammonia formation and that after 4 hours of preservation without irradiation was negligible.

Ammonia was estimated by the standard method of Folin. The slight precipitate produced by the addition of a few drops of 10 % sodium tungstate solution to the R.G.B., followed by acidification with HCl to Congo red, was centrifuged out, and, after making alkaline with Na_2CO_3 solution, the ammonia was displaced by a current of air into 5 ml. of $N/20$ HCl and estimated colorimetrically by Nessler's reagent. Preliminary tests with known amounts of ammonia showed a recovery of 95–97 % by the aeration process.

The rate of ammonia formation is expressed in units similar to those used for respiration and glycolysis.

$$Q_{NH_3} = \frac{\mu l.\ NH_3}{\text{mg. dry tissue} \times \text{hours}}.$$

Tables I and II include a selection of results obtained after irradiation of tumour and normal tissues respectively.

Table I. *Increased rate of ammonia formation by J.R.S. tissue irradiated under different conditions.*

Time of irradiation hr.	Temp. during irradiation ° C.	Irrigating medium used during irradiation	Irradiated Q_{NH_3}	Control Q_{NH_3}	Increase in Q_{NH_3} due to irradiation %
4	0–5	R.G.B. aerobic	1·10	0·34	220
4	0–5	„	0·87	0·40	120
4·5	0–5	„	0·75	0·32	130
3	0–5	„	0·71	0·36	100
4	0–5	„	0·97	0·66	50
4	0–5	„	0·91	0·49	85
4·5	0–5	„	0·86	0·50	70
4	37·8	„	0·94	0·45	110
4	37·8	„	0·91	0·60	50
4	37·8	„	1·10	0·61	80
4	37·8	„	0·86	0·55	55
3·5	37·8	*M*/600 HCN in R.G.B.	1·10	0·49	125
3·5	37·8	„	1·32	0·81	65
4	37·8	„	0·62	0·41	50
4	37·8	R.G.B. anaerobic	0·80	0·41	100
4	37·8	„	0·71	0·45	60

Table II. *Increased rate of ammonia formation by irradiated normal tissues.*

Time of irradiation = 4 hr.
Temperature during irradiation = 0–5°.

Rat tissue	Irradiated Q_{NH_3}	Control Q_{NH_3}	Increase in Q_{NH_3} due to irradiation %
Brain	0·87	0·54	60
	0·94	0·71	32
	0·41	0·24	70
	1·35	0·66	105
	0·60	0·38	58
Liver	0·34	0·26	30
	0·40	0·28	43
	0·46	0·31	48
	0·23	0·22	—
Testis	0·37	0·15	145
	0·36	0·16	125
	0·12	0·07	40
	0·26	0·16	63

The first seven results in Table I were obtained with tumour tissue in which the glycolytic process was largely suppressed after irradiation at low temperature, and, it was thought, illustrated the relationship existing between glycolysis and ammonia formation described by Warburg *et al.* [1924]. The rate of ammonia formation was of the same order whether the final incubation was carried out in the presence or absence of glucose, owing to the inactivation of the glycolytic system.

The succeeding experiments, in which radiation was applied at 37·8° under different environmental conditions, and in which glycolysis remained active, showed that the earlier conception was wrong. Under these conditions, excessive ammonia formation was not coupled with glycolytic failure, but, to judge from earlier work [Crabtree, 1932], with incipient or actual respiratory failure. Ammonia was formed at a similarly increased rate in media with or without glucose, i.e. with glycolysis proceeding actively or not at all. These experiments make it clear that an increased rate of ammonia formation is a characteristic reaction induced by radiation itself and independent of the glycolytic system. The experiments with normal tissues shown in Table II confirm this. It is noteworthy that the rate of ammonia production in these normal tissues varies considerably and may be determined by variations in their functional activity or the age of the animals used. The increased rate of ammonia production induced by radiation is, however, a common and significant factor. Taken in conjunction with the earlier findings that carbohydrate metabolism is primarily damaged by radiation, and assuming that this excess ammonia formation represents increased protein breakdown, it would be anticipated that a fall of respiratory quotient would occur as the damaging effects of radiation progressed. It may be recalled that Bancroft *et al.* [1935] recorded that X-radiation treatment *in vivo* led to a fall of respiratory quotient in the Philadelphia rat sarcoma No. 1.

SUMMARY AND CONCLUSIONS.

1. Irradiation of tissues *in vitro* induces an increased rate of ammonia formation.
2. In the case of tumour tissues this effect is independent of the state of activity of the glycolytic system. Whether the latter is suppressed by irradiation at low temperature or in full activity after irradiation at body temperature, ammonia production increases to a similar extent.
3. Normal non-glycolysing tissues—liver, testes, brain—also produce ammonia more rapidly after irradiation.
4. It is suggested that this increased ammonia production is due to the secondary utilization of protein as carbohydrate metabolism, either oxidative or splitting, is impaired.

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CCXCIX. THE MOLECULAR STRUCTURE OF GLYCOGEN FROM THE WHOLE TISSUES OF *MYTILUS EDULIS.*

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THE investigation, by Haworth's "end-group assay" method, of glycogen obtained by the alkaline disintegration of the whole tissues of *Mytilus edulis* is described below. Employing the author's technique [Bell, 1935; 1936] the chemical basic molecule of this glycogen is shown to be derived from 18 glucose units. The compositions of glycogens already investigated in this way are listed in Table I.

Table I.

Source of glycogen	No. of glucose units	Reference
Rabbit liver	12	Haworth & Percival [1932]
Rabbit liver	12	Bell [1935]
Rabbit liver (commercial)	18	Haworth [1935]
Rabbit liver, after galactose feeding	18	Bell [1936]
Fish liver	12	Bell [1935]

The *Mytilus* glycogen, examined according to Bell & Young [1934] and Bell & Kosterlitz [1935], showed no significant differences from the other glycogens described by these authors. Similarly the methylated and acetylated derivatives differed in no apparent manner from the corresponding substances prepared from glycogens from other sources (see Table I).

Hydrolysis of the methylated glycogen, followed by separation and identification of the cleavage products, yielded tetramethylglucose amounting to 6 % of the starting material, corresponding to a mean "chain-length" of 18 glucose units for the glycogen molecule. This chain length is further confirmed by the relative amounts of the different cleavage products, isolated in over 90 % yield, viz.:

1 mol. ... ... 2:3:4:6-tetramethylglucose.
15 mols. ... ... 2:3:6-trimethylglucose.
2 mols. ... ... dimethylglucoses.

It is noteworthy that here, and in the investigations cited above, glycogen appears to occur solely in one or other of the two known forms, 12-unit or 18-unit, and that we have as yet no evidence for the occurrence of mixtures of these.

EXPERIMENTAL.

The crude glycogen was prepared by the Pflüger method by Dr F. G. Young, to whom the author is indebted for the material used in this investigation. Purified by the procedure of Bell & Young [1934], the glycogen showed a rate of hydrolysis with aqueous acid identical with that of previously examined specimens. With iodine, the colour was identical with that developed by fish liver glycogen.

Mytilus glycogen had the following other properties: $[\alpha]_D^{25°}$ in water, +195·4° ($l=2$, $c=0·5$); reducing power (Macleod-Robison, glucose = 100), 1·3; phosphate, inorganic—nil, organic—nil.

Acetylation. By the method of Haworth & Percival [1932] 30 g. of glycogen gave 48 g. acetate (90 % of theory); $[\alpha]_D^{25°}$ in $CHCl_3$, +172°. (Found, $COCH_3$: 44·5 %.)

Methylation. 37 g. of the acetate simultaneously deacetylated and methylated by the procedure of the above authors, yielded, after 12 treatments, 24·0 g. (90 %) of methylated glycogen. (Found, OMe: 45·1 %.) $[\alpha]_D^{20°}$ in $CHCl_3$, +211° ($l=2$, $c=4·5$).

Hydrolysis by aqueous acid and separation of cleavage products.

The procedure was exactly as described by the author [Bell, 1935; 1936] (Table II).

Table II.

OMe % of methylated glycogen	45·1
Amount hydrolysed (g.)	22·95
2:3:4:6-Tetramethylglucose found (g.)	1·359
2:3:4:6-Tetramethylglucose as % of starting material	6
2:3:4:6-Tetramethylglucose M.P. drained on tile	87°
2:3:4:6-Tetramethylglucose $[\alpha]_D$ in water ($c=5$)	+82·0°
2:3:4:6-Tetramethylglucose n_D^{17}	1·4585
2:3:4:6-Tetramethylglucose OMe %	52·4
2:3:6-Trimethylglucose found (g.)	18·79
2:3:6-Trimethylglucose M.P. drained on tile	115°
2:3:6-Trimethylglucose $[\alpha]_D$ in water ($c=4·5$)	+70·3°
Dimethylglucose found (g.)	2·19

The trimethylglucose fraction consisted entirely of the 2:3:6-derivative, and no evidence of monomethyl- or unmethylated glucose was found in the hydrolysate of the methylated glycogen.

The methyl content and the ratios of the different fractions to one another correspond well with the methylated glycogen being an octadecasaccharide in which 54 of the 56 free OH groups are methylated [cf. Bell, 1936].

Table III.

Ratios	Found	Calculated
$\frac{\text{Trimethylglucose}}{\text{Tetramethylglucose}}$	13·8	13·1
$\frac{\text{Trimethylglucose}}{\text{Dimethylglucose}}$	8·6	7·4

The author is indebted to Sir F. G. Hopkins for his encouragement, to Mrs Lutwak-Mann for carrying out phosphate determinations and to the Medical Research Council for a grant which partly defrayed the expenses of this work.

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CCC. THE ANALYSIS OF CARBOHYDRATES OF THE CELL WALL OF PLANTS.

II. THE DETERMINATION OF PENTOSES AS SINGLE SUBSTANCES AND IN MIXTURES CONTAINING URONIC ACIDS AND HEXOSES.

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In the first communication of this series [Norris & Resch, 1935] the importance of the accurate determination of the furfuraldehyde obtained from plant materials was demonstrated, and the relationship between uronic acids and the furfuraldehyde yielded by them was placed on a more satisfactory basis.

Whilst the above work was in progress and for some time following its publication, a critical survey of the methods available for the determination of furfuraldehyde was undertaken, and we now believe that the proximate analysis of plant materials is susceptible of greater accuracy than was formerly the case.

In this communication it is proposed to describe only investigations on the well-known method of Kröber [1901], which employs phloroglucinol as precipitating agent.

The inaccuracies which have become associated with the determination arise from a number of errors and inconsistencies in applying the original method. The duration and temperature of distillation and the apparatus used for it must be standardized, as also the conditions of precipitation and after-treatment of the precipitate. Strict adherence to a fixed set of conditions is essential.

In many cases the original method of Kröber has not been exactly followed, but the subsequent calculation has been made on the basis of Kröber's tables or formulae. This is particularly to be noted where the precipitated phloroglucide has been washed with alcohol; it is quite indefensible to use Kröber's tables in such cases, since treatment with alcohol was not included in his method.

Again, even supposing that the use of Kröber's results is permissible, there is inherent inaccuracy in the employment of formulae based on them. The formula, $F=(P+0{\cdot}0052)\times0{\cdot}5185$ (where F=furfuraldehyde and P=phloroglucide), is often used and is generally regarded as accurate within the limits $P=0{\cdot}03$ to 0·3 g. Analysis of Kröber's tables will show that this is not, in fact, the case: the relationship between furfuraldehyde and phloroglucide over the range indicated can be expressed graphically only by a slightly curved line, and the straight line relationship indicated in the formula is only an approximation and is only valid over a very small range.

We have therefore based our investigation on the premise that if the distillation and precipitation procedures are really standardized, then a given weight of pentose will correspond to a definite weight of phloroglucide, within the limits of experimental error, and that the relationship between product and phloroglucide may, over a small range of weights only, be represented by a straight line. The equation best expressing the relationship has been calculated by the method of least squares and the standard error of experiment determined mathematically.

In this manner equations have been derived to indicate the relationship between phloroglucide and (i) furfuraldehyde; (ii) arabinose; (iii) xylose; (iv) galacturonic acid; (v) pectolic acid; (vi) glycuronic acid (as euxanthic acid).

The greatest source of error in modern usage of the Kröber method, and one which has apparently been largely overlooked, lies in the fact that in actual practice the investigator rarely, if ever, deals with single substances, but is concerned almost invariably with mixtures of greater or less complexity. The tacit assumption that the weight of phloroglucide produced by a number of substances in admixture exactly corresponds to the sum of the weights of phloroglucide produced by each separately is shown in this communication to be unjustified, nor does Kröber give any indication that his tables may be used for any but pure single substances.

To establish relationships for the large number of possible mixtures of two or more of the commonly occurring pentoses, uronic acids and hexoses, constitutes a laborious routine and requires a statistical investigation whose results would doubtfully justify the time expended. We have therefore confined ourselves to the examination of those mixtures commonly met with in cell wall chemistry and have outlined methods which may be employed for the more accurate analysis of such substances as pectin, the hemicelluloses etc.

EXPERIMENTAL.

Distillation. In the first instance the apparatus described in the previous communication [Norris & Resch, 1935] was used, but all the joints involving rubber bungs were replaced by interchangeable ground glass connexions. This involved a change in the shape and diameter of the side tube leading to the condenser, and it was found necessary to "lag" this by means of asbestos paper. Also, in order to maintain the normal rate of distillation it was necessary to increase the temperature of the glycerol-bath to 175–180°. Comparative results using the old and the new forms of apparatus did not invariably show the superiority of the latter, but from every practical point of view the ground glass joints are preferable, and any remaining doubts as to the possible effect of decomposition of the rubber bungs in the older form are removed.

In order to maintain a more even distillation temperature and to avoid possible violent fluctuations in the concentration of the acid it was decided to revert to the original method of Kröber, in that the acid is now added in portions of 30 ml. every 10 min. instead of 60 ml. every 20 min. as hitherto.

Experiments with pure furfuraldehyde by direct precipitation and by precipitation after the usual distillation showed that there is a slight loss of furfuraldehyde—about 1 %—during distillation. In order to remove furfuraldehyde from the influence of the acid as quickly as possible and to obviate possible oxidation, nitrogen was bubbled through the apparatus in several series of experiments. It was thought possible that the current of nitrogen might increase evaporation of furfuraldehyde at the delivery tube and to obviate this the tube was run directly into the solution of phloroglucinol in some experiments. The use of nitrogen brought no increase in yield, however, and was discontinued. There seems little doubt that, since in the case of furfuraldehyde itself the distillation loss is within the normal limits of experimental error, the low yields observed in the case of the pentoses are due to more deep-seated degradation of the sugar molecule itself, depending upon differences in configuration [cf. Bott & Hirst, 1932].

Precipitation. The results of further experiments with phloroglucinol as precipitant have involved very little modification in the procedure previously described. It was established that, although the precipitate is collected in a thin layer in the sintered glass crucibles, 100 ml. of water for washing are insufficient, and we have now adopted 150 ml. as standard.

The practice of washing the precipitate with hot alcohol has been entirely abandoned. Statistical examination of the results after such washing has shown that the supposed beneficial effects on concordancy are illusory.

The hygroscopic nature of the precipitate has often been observed and means of obviating difficulties in weighing suggested. The practice of placing the crucibles in tared weighing bottles appeared on test to be of doubtful value, since large weighing bottles are necessary and the effect of moisture on the glass surface under varying conditions of humidity is appreciable. It is possible to form a close estimate of the weight of the crucible and contents and to place most of the weights on the balance pan before removing the crucible from the desiccator. Using the open crucible in this way it was found possible to complete the weighing within 60 sec., and no disturbing effects due to absorption of moisture have been observed.

Standard method. The final method adopted was essentially similar to that previously described, but with the following changes: (i) interchangeable ground glass connexions replace rubber bungs; (ii) the temperature of distillation is now 175–180° instead of 170°; (iii) additions of 12 % hydrochloric acid at the rate of 30 ml. every 10 min.; (iv) the precipitate is washed with 150 ml. of cold distilled water instead of 100 ml.

Relation between phloroglucide and furfuraldehyde-yielding substances.

1. *Furfuraldehyde.* For the purpose of these experiments specially purified furfuraldehyde was obtained from the manufacturers and then submitted to five distillations. The final product gave the correct boiling-point and refractive index and was directly distilled into the weighing bottle to avoid any possible oxidation. A known weight was made up to volume in 12 % hydrochloric acid and aliquot portions were precipitated direct as usual with phloroglucinol. By omitting the distillation procedure in this case we were able to ascertain the absolute relationship between furfuraldehyde and phloroglucide obtained by the usual precipitation method.

Table I. *Relation between furfuraldehyde and phloroglucide.*

Wt. of furfuraldehyde g.	Wt. of phloroglucide g.	Wt. of furfuraldehyde g.	Wt. of phloroglucide g.
0·0283	0·0525	0·0532	0·1015
0·0322	0·0606	0·0538	0·1029
0·0366	0·0686	0·0556	0·1064
0·0400	0·0755	0·0595	0·1145
0·0405	0·0765	0·0634	0·1229
0·0506	0·0981		

2. *Arabinose.* Purified specimens of arabinose were used in this series, aliquot portions of a standard solution being distilled and precipitated according to the standard procedure. The arabinose was calculated to anhydroarabinose,

since this is the more convenient form in practical applications. These remarks apply also to the other sugars dealt with.

Table II. *Relation between anhydroarabinose and phloroglucide.*

Wt. of anhydroarabinose g.	Wt. of phloroglucide g.	Wt. of anhydroarabinose g.	Wt. of phloroglucide g.
0·0624	0·0624	0·0946	0·0963
0·0719	0·0725	0·1135	0·1179
0·0757	0·0758	0·1270	0·1316
0·0930	0·0952		

3. *Xylose.* This was treated in a precisely analogous manner to arabinose.

Table III. *Relation between anhydroxylose and phloroglucide.*

Wt. of anhydroxylose g.	Wt. of phloroglucide g.	Wt. of anhydroxylose g.	Wt. of phloroglucide g.
0·0566	0·0649	0·0811	0·0953
0·0697	0·0812	0·0937	0·1100
0·0704	0·0821	0·0995	0·1183

4. *Galacturonic acid* and 5. *pectolic acid.* The yields of phloroglucide from these sources are given in the previous communication [Norris & Resch, 1935, p. 1593] and need not be repeated here. The results have been submitted to statistical analysis together with those obtained for the other products in the present communication (*vide infra*).

6. *Glycuronic acid* (as euxanthic acid). In this case the results quoted in the earlier communication cover too narrow a range for present purposes. A second series of experiments gave the results shown in Table IV, wherein the euxanthic acid is calculated to carbon dioxide. Uronic anhydride is readily obtained by multiplying by 4.

Table IV. *Relation between carbon dioxide from glycuronic anhydride and phloroglucide.*

CO_2 equivalent of urone g.	Wt. of phloroglucide g.	CO_2 equivalent of urone g.	Wt. of phloroglucide g.
0·0209	0·0275	0·0417	0·0594
0·0261	0·0353	0·0438	0·0636
0·0313	0·0432	0·0459	0·0640
0·0365	0·0517	0·0469	0·0666

Interpretation of results.

The foregoing results were subjected to mathematical analysis in two ways: (*a*) by the method of least squares the linear equation which best represented the relationships between product and phloroglucide was calculated; and (*b*) the standard error of the determinations was computed. In each case, then, it was possible to establish a linear relationship indicated by the following general equation:

$$S=a+bP\pm e,$$

where S = substance under examination;
P = phloroglucide;
e = standard error of the determination;
a and b = constants.

The following equations were thus derived:

(1) Furfuraldehyde: $F = 0{\cdot}5030\ P + 0{\cdot}0018 \pm 0{\cdot}0003$.

(2) Anhydroarabinose: $A = 0{\cdot}9253\ P + 0{\cdot}0050 \pm 0{\cdot}0007$.

(3) Anhydroxylose: $X = 0{\cdot}7673\ P + 0{\cdot}0069 \pm 0{\cdot}0009$.

(4) Galacturonic anhydride, as carbon dioxide:
$$C_{Ga} = 0{\cdot}5778\ P + 0{\cdot}0013 \pm 0{\cdot}0009.$$

(5) Pectolic anhydride, as carbon dioxide:
$$C_p = 0{\cdot}5744\ P + 0{\cdot}00076 \pm 0{\cdot}0008.$$

(6) Glycuronic anhydride, as carbon dioxide:
$$C_{Gy} = 0{\cdot}6613\ P + 0{\cdot}0026 \pm 0{\cdot}0006.$$

It will be observed that the smallest experimental error occurs in the first equation, a result to be anticipated, since the furfuraldehyde was determined directly, whereas all the other experiments involved distillation. It was shown by statistical methods that there is no significant difference in the yield of phloroglucide from galacturonic acid itself as compared with that from pectolic acid, a confirmation of the result obtained by inspection in the previous paper. It should be emphasized that the equations given are only valid for the range of weights indicated in the tables, which have been chosen to include the weights commonly dealt with by us in applications to cell wall substances. Furthermore, it has been repeatedly noticed by us that different workers in these laboratories invariably obtain slightly different equations based on their own tables of results, and the standard errors also show slight differences similarly. It is possible that the differences observed are not of great significance (in the mathematical sense of the term), but it seemed desirable to make this point as an indication that for completely satisfactory results each worker should prepare his own experimental data.

Application to the analysis of mixtures.

The statistical method was now applied to the case of mixtures of substances commonly met with in dealing with pectin and the hemicelluloses. In the former case mixtures containing arabinose, galacturonic acid and galactose occur, and the first set of experiments below was designed to enable us more accurately to analyse such mixtures.

Arabinose and galacturonic acid (as pectolic acid). Pectolic acid was used in these experiments since it is more readily prepared than galacturonic acid, and moreover approximates much more closely to the form in which galacturonic

Table V.

Anhydro-arabinose g.	CO_2 equivalent to pectolic acid g.	Phloroglucide Found g.	Phloroglucide Calc. g.
0·0238	0·04943	0·1064	0·1050
0·0476	0·04943	0·1282	0·1307
0·0714	0·04943	0·1554	0·1565
0·0220	0·05830	0·1253	0·1186
0·0476	0·05698	0·1393	0·1439
0·0714	0·05698	0·1663	0·1697
0·0238	0·06575	0·1354	0·1334
0·0476	0·06575	0·1614	0·1591
0·0714	0·06575	0·1863	0·1849

acid occurs under normal conditions. Mixtures were prepared according to the requirements of a "Latin square" and were then submitted to the standard distillation and precipitation procedures. From the results obtained an equation correlating anhydroarabinose, the carbon dioxide equivalent of pectolic acid and the phloroglucide produced was calculated.

The equation deduced from the results (first three columns) was:

$$(7)\quad A = 0{\cdot}9942\,P - 1{\cdot}9081\,C_p + 0{\cdot}01313 \pm 0{\cdot}00305.$$

It will be noted that the experimental error in the case of these mixtures is considerably greater than those for the substances treated separately (equations (2) and (5)). The figures in the final column were calculated from the individual equations quoted above, and these show quite clearly that the phloroglucide obtained from the mixtures is not the sum of the quantities obtained from the single substances. The differences observed are greater than the experimental error and therefore of definite significance.

In analysing such mixtures, then, it is possible to derive the weights of the two constituents present with greater accuracy than heretofore, since by the usual method of decarboxylation [Dickson *et al.* 1930] the amount of uronic anhydride may be obtained and, following a phloroglucide determination, the value of C_p may be substituted in the equation, whence the value of A may be calculated. The equation, however, is not necessarily valid outside the range of weights from which it was derived.

Pectolic acid and galactose. Apart from the alleged production of small amounts of ω-hydroxymethylfurfuraldehyde, galactose should make no contribution to the total phloroglucide produced from mixtures in which it occurs. At the same time it is possible that it may have some influence on the yield of phloroglucide normally obtained from the usual furfuraldehyde-yielding substances. This and the following set of experiments were accordingly designed to determine whether or not galactose had a significant effect.

Table VI.

Galactose g.	CO_2 equivalent of pectolic acid g.	Phloroglucide g.
0·0221	0·06575	0·1121
0·0442	0·06575	0·1123
0·0663	0·06575	0·1114
0·0736	0·06555	0·1155
0·0819	0·07135	0·1251
0·1604	0·06983	0·1196
0·1839	0·07963	0·1351
0·2045	0·05935	0·1051
0·2157	0·06253	0·1106
0·2298	0·06643	0·1168

These results are discussed below.

Arabinose, pectolic acid and galactose. **It remains to examine the effect of galactose when present in mixtures containing arabinose in addition to pectolic acid. To this end mixtures were prepared and analysed as shown in Table VII.**

Analysis of these results, and those of Table VI, leads to the same conclusion, namely, that galactose exerts an effect on the yields of phloroglucide normally to be expected from the other constituent or constituents of the mixture, but that such effect is not significant until the proportion of galactose reaches more than

Table VII.

Anhydro-arabinose g.	CO_2 equivalent of pectolic acid g.	Galactose g.	Phloroglucide g.
0·0220	0·05830	0·0205	0·1253
0·0220	0·06748	0·0205	0·1413
0·0238	0·06575	0·0221	0·1379
0·0476	0·06575	0·0221	0·1647
0·0238	0·06575	0·0442	0·1353

50 % of the total constituents. In general it may be said that where galactose is present in small proportions there is a slight relative increase in phloroglucide yield, but where the proportion is greater there is a slight relative decrease. Since it is not usual to work with mixtures containing more than 50 % of galactose, at least in pectin chemistry, its effect may usually be neglected.

Thus, in dealing with ternary mixtures of the above type, uronic acid may be determined by quantitative decarboxylation, arabinose may be calculated as already described (equation (7)) from the phloroglucide determination and galactose obtained by difference.

Xylose and glycuronic acid (as euxanthic acid). Mixtures in the xylose, glycuronic acid, glucose series were examined in a similar manner to that already described. The relation between xylose, glycuronic acid and phloroglucide was first determined in accordance with the results in Table VIII.

Table VIII.

Anhydroxylose g.	CO_2 equivalent of glycuronic acid g.	Phloroglucide g.
0·0660	0·0021	0·0797
0·0660	0·0042	0·0838
0·0660	0·0078	0·0869
0·0660	0·0115	0·0926
0·0660	0·0156	0·0996
0·0704	0·0083	0·0943
0·0792	0·0021	0·0951
0·0792	0·0042	0·0990
0·0792	0·0078	0·1043
0·0968	0·0021	0·1189
0·0968	0·0042	0·1217
0·0968	0·0078	0·1271

The equation derived from these results was:

$$(8)\quad X = 0{\cdot}7859\,P - 1{\cdot}146\,C_{G_y} + 0{\cdot}0060 \pm 0{\cdot}0006.$$

In this instance, also, the yield of phloroglucide obtained from the substances in admixture was found not to coincide with the sum of the yields of the constituents taken separately. The yield of "mixed" phloroglucide is always higher than might be anticipated. The equation may be used for calculating xylose-glycuronic acid mixtures since C_{G_y} may be determined independently.

Xylose and glucose. The effect of addition of varying proportions of glucose to xylose on the phloroglucide yield was next examined as in Table IX.

The figures of the final column were derived from equation (3) and it was shown by statistical treatment that the differences between found and calculated values were not significant, i.e. the effect of glucose on the furfuraldehyde yield of xylose may be neglected for the range of weights taken.

Table IX.

Anhydroglucose	Anhydroxylose	Phloroglucide	
		Found	Calc.
g.	g.	g.	g.
0·0270	0·0748	0·0860	0·0885
0·0540	0·0748	0·0860	0·0885
0·0765	0·0748	0·0876	0·0885
0·1530	0·0748	0·0877	0·0885
0·3060	0·0748	0·0886	0·0885
0·0720	0·0704	0·0842	0·0828
0·0360	0·1056	0·1253	0·1286
0·0720	0·1056	0·1266	0·1286
0·1080	0·1056	0·1252	0·1286

It may be mentioned in this case that the precipitates obtained were submitted to alcohol washing in order to eliminate ω-hydroxymethylfurfuraldehyde phloroglucide if any were present. However, the results obtained confirmed our dissatisfaction with the procedure, since they showed a considerable and variable depression in the yield, with consequently a much larger standard error of experiment.

Xylose, glycuronic acid and glucose. The yield of phloroglucide from the mixtures of all the substances is shown in Table X, together with the weights of xylose calculated from equation (8).

Table X.

Anhydroxylose		CO_2 equivalent of urone	Anhydro-glucose	Phloro-glucide
Taken	Calc.			
g.	g.	g.	g.	g.
0·0704	0·0709	0·0083	0·0360	0·0947
0·0704	0·0722	0·0083	0·0720	0·0963
0·0704	0·0722	0·0083	0·1080	0·0963
0·0704	0·0728	0·0042	0·0720	0·0910
0·0704	0·0717	0·0125	0·0720	0·1019
0·0704	0·0732	0·0167	0·0720	0·1098

The differences between the weights of xylose taken and those calculated are in each case positive, and they are significant as shown by statistical analysis. The presence of glucose in the ternary mixture thus involves results which are not in accord with equation (8) obtained for xylose-euxanthic acid mixtures, and the case is by no means so clear-cut as the previous one involving arabinose, pectolic acid and galactose.

It would be possible to arrange a series of experiments so as to enable an equation relating phloroglucide with the three constituents to be calculated: $X=a+bP+cC_{Gy}+dG$; this would not be of much value, since although P and C_{Gy} could be determined we should still have an equation involving the two unknowns X and G. Since there is no satisfactory way of determining glucose in such a mixture, the values of X and G would remain indeterminate.

It may be possible, when experiments now proceeding with other furfuraldehyde precipitants are complete, to derive a second equation relating, for example, the thiobarbituric acid precipitate with the three constituents; in which case we might, by calculating two simultaneous equations in X and G, obtain values for these constituents. It is hoped to make an additional communication on this point.

For the present, however, in analysing such mixtures [Angell & Norris, 1936], the most satisfactory procedure appears to lie in calculating xylose and glycuronic acid from equation (8), obtaining glucose by difference. The approximate composition being known in this manner, a correction is then applied to the weight of phloroglucide by comparison with results in Table X for a mixture of approximately similar constitution. Recalculation then follows as above. Whilst this gives a final result which is still only an approximation, there is little doubt that it represents a considerable improvement on results hitherto obtained.

In conclusion, it may be stated that the methods described in this communication have been applied to hemicelluloses [Angell & Norris, 1936] and pectin [Norris & Resch, in preparation], and that by their means much more satisfactory results have been obtained. Except in the former case mentioned, no assumptions are made, the results by standard methods being mathematically treated as general cases and later applied to particular ones met with in practice. In our view, individual standardization is essential in furfuraldehyde determination, and the method and apparatus require as close control as, for example, the well-known Reichert-Wollny determination.[1]

Summary.

1. Criticisms of the usual procedure in furfuraldehyde determination are made and remedies suggested.

2. A standardized procedure based on experiment is described.

3. The relation between phloroglucide and furfuraldehyde-yielding substances, singly and in admixture, is determined.

4. The effect of the presence of galactose and glucose in such mixtures is also determined.

5. The results are treated mathematically, equations indicating the relationships and standard errors of experiment being deduced.

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[1] It has not been possible in this contribution to include details of mathematical analysis, but these can be provided on request.

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CCCI. STUDIES ON HEMICELLULOSES.

V. ADDITIONAL NOTES ON THE HEMICELLULOSES OF MAIZE COBS.

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THE method of fractionation of the hemicelluloses of cell wall substances proposed by Norris & Preece [1930] would appear to have been adopted with few major modifications by other workers in this field. Briefly, the sodium hydroxide extract of the cell wall material is precipitated in three stages: by addition of acetic acid; half a volume of acetone; and one volume of acetone. Each of these fractions may again be subdivided by treatment with Fehling's solution, those fractions recovered from the insoluble gel being designated A_1, B_1, C_1, whilst those obtained from the copper solution are referred to as A_2, B_2, C_2.

In order to avoid confusion in questions of classification raised, for example, by Buston [1935], it seems desirable to emphasize the fact that the systematic designations indicate not definite classes of hemicellulose but particular methods of preparation. It has never been suggested that any one fraction represents a particular hemicellulose of definite chemical entity or that hemicelluloses of the same designation but prepared from different plant sources are necessarily chemically identical. The fractionation is very largely physical and it is more than probable that the several fractions usually obtained still comprise more or less complex mixtures.

Evidence obtained during the course of experiments on the hemicelluloses of the flowers of the hop, described in a subsequent paper of this series, would appear to indicate that in the past the precipitation at some stages has been incomplete, and especially is this the case where the first fraction, precipitable by acetic acid, is concerned. Thus, in precipitating fraction A from the NaOH extract, the original directions proposed addition of glacial acetic acid until the mixture was acid, but the actual amount necessary was not specified. It has been found that the *p*H of precipitation has a considerable effect on the amount of hemicellulose precipitated, and it will be appreciated that a considerable volume of acetic acid is necessary in order to lower the *p*H of the mixture to any extent, since a buffered sodium acetate-acetic acid mixture is produced. It seems very probable that in the preparation of maize cob hemicelluloses [Preece, 1930] a considerable proportion of fraction A escaped precipitation at the acid stage and appeared later as fractions B and C.

Maize cobs were chosen for the present investigation since they were most readily available, but there is reason to believe that the *p*H of the solution at precipitation is of general importance. Thus the precipitability of hemicelluloses from hops was found to depend on the *p*H of the medium and, moreover, the optimum *p*H in this case differed from that in the case of the maize cob products. It is, in fact, conceivable that the *p*H of minimum solubility of the hemicelluloses is as definite a physical characteristic as the isoelectric point of proteins.

The precipitation of the fractions by copper solutions was also examined in the case of maize cob hemicelluloses. The solutions used were Fehling's solution, Schweitzer's solution and a mixture of glycerol and cupric sulphate. For the decomposition of the gels both dilute hydrochloric and acetic acids were used. It was found that the mixture of glycerol and cupric sulphate was the most effective precipitant, whilst for the decomposition of the gel, acetic was to be preferred to hydrochloric acid.

EXPERIMENTAL.

Precipitation of hemicellulose A_1: effect of pH on yield.

In a preliminary experiment the hemicellulose A_1 prepared by Preece [1930] was used in 1 % solution in 4 % NaOH. 5 ml. portions of this solution in a series of test-tubes were made up to 20 ml. with distilled water and acetic acid in amounts sufficient to bring the solutions to a range of *p*H values varying from 7 to 3 at intervals of 0·5 (determined colorimetrically). Turbidity was observed in each tube almost immediately, but on standing overnight the precipitate settled out completely in only one tube, namely that whose contents were adjusted to *p*H 4. On further standing, precipitates in the other tubes settled out gradually, but the most complete precipitation was still obtained at *p*H 4.

The results of the preliminary qualitative study were confirmed by quantitative experiments on a larger scale. In this case, the maize cobs were treated in the usual manner for the preparation of a hemicellulose extract in 4 % NaOH. 500 ml. portions of the extract were used and made up with acetic acid and distilled water to 800 ml. so as to give a series as before, each mixture at a different *p*H. The *p*H was determined electrometrically, using the quinhydrone electrode. The mixtures were allowed to stand for 7 days, after which the precipitates were filtered off, washed free from acid by 50 % alcohol, dried as usual and weighed. Each sample contained about 1 % lignin and ash varying between 1·4 and 2·9 %. The yields obtained, on a lignin- and ash-free basis, were as follows:

*p*H at precipitation	Yield (g.)
6·0	11·6
5·4	11·7
4·7	12·4
4·1	14·2
3·7	14·6

The increased amount precipitated at *p*H 3·7 as compared with that at 4·1 is counterbalanced by a considerably increased difficulty in filtration. On the large scale it was found to be exceedingly difficult to separate the precipitate obtained at *p*H 3·7 even at the centrifuge, whereas that obtained at 4·1 presented no such difficulty. It was therefore concluded that the optimum *p*H for the precipitation of the hemicellulose was 4·0–4·1.

Composition of the precipitates.

It was found that there was no significant difference in the yields of furfuraldehyde from the precipitates obtained at different *p*H values, and hence the increased yields observed represent definite improvements in yield of the same substance or mixture of substances. A typical sample dried to constant weight at 98° contained 1·72 % ash and 1·26 % lignin. Uronic anhydride and furfuraldehyde were determined, and the percentage of xylan, X, was calculated from the formula $X = 0{\cdot}0060 + 0{\cdot}7859P - 1{\cdot}146C$, where P = phloroglucide and C = carbon dioxide from uronic anhydride. [Norris & Resch, 1935; Angell *et al.*, 1936.]

In this way the composition was found to be:

	%
Xylan	94·8
Glycuronic anhydride	5·1
Methoxyl	0·5

Now the yield of this fraction A_1 obtained by precipitation at pH 4 corresponded to 14·5 % of the dry maize cobs. For comparison we may quote the results obtained by Preece [1930]:

Fraction	Yield %	Methylpentosan %	Furfuraldehyde %	Uronic anhydride %
A_1	8·0	Nil	61·49	3·76
B_1	6·0	7·01	57·31	5·20
C_1	1·0	7·42	54·57	7·40
C_2	0·5	8·91	43·90	4·56

It will be seen that our yield of fraction A_1 accounts for Preece's fractions A_1, B_1 and possibly C_1. Each of these latter fractions was closely similar in composition, consisting chiefly of xylan and glycuronic anhydride. We have been unable to confirm the presence of methylpentosan suggested by Preece. This suggestion was based largely on the fact that about 7 % of the phloroglucide was soluble in hot alcohol, giving a brownish solution. We also have found that the alcoholic solution varied in colour from green to brown, but in no case was the absorption band characteristic of methylfurfuraldehydephloroglucide detected. Furthermore, Rosenthaler's [1909] reaction gave negative results on the hemicellulose and its hydrolysis products.

Thus, Preece's fractions A_1, B_1 and C_1 are in reality one fraction only, A_1; fraction C_2 appears to have a separate existence since in this case the xylan is replaced by araban.

Precipitation by copper solutions.

The sample of hemicellulose A_1 used in these experiments contained 2·85 % of ash and 0·94 % of lignin. A solution was prepared containing 1 % (on an ash-free basis) of the hemicellulose in 4 % NaOH. 200 ml. portions of the solution were measured out, precipitated with a copper solution and decomposed with acid, the following reagents being used:

For precipitation: (*a*) 10 ml. glycerol followed by 20 ml. *M* cupric sulphate; (*b*) 60 ml. of Fehling's solution; (*c*) 30 ml. of Schweitzer's reagent.

For decomposition: (*d*) 200 ml. 25 % acetic acid; (*e*) 200 ml. *N* HCl.

In each case, after addition with stirring of the precipitating reagent, the solution was allowed to stand overnight.

The copper gel was then filtered off on a hardened paper and subsequently decomposed with 200 ml. of acid, the solution being stirred mechanically. Half a volume of acetone was added to the acid solution and after standing overnight the precipitate was filtered off on a Berlin funnel, washed with 50 % acetone until free from acid and copper and dried to constant weight as usual. The following yields (ash-free) were obtained:

Precipitation by	Decomposition by	Yield g.	Yield %	Ash %
Glycerol and cupric sulphate	HOAc	1·75	87·2	1·20
" "	HCl	1·54	76·8	0·60
Fehling's solution	HOAc	1·43	71·0	0·73
"	HCl	1·39	69·0	1·00
Schweitzer's reagent	HOAc	1·42	70·8	2·64

As in the case of the acid precipitations, the chemical composition of all the products was identical within the usual experimental error, so that the larger yields in the case of glycerol and cupric sulphate represent genuine improvements in technique.

Buston [1934], working with a hemicellulose mixture from cocksfoot grass, commented on considerable losses of hemicellulose when precipitating fraction A and when carrying out the copper precipitation. It seems quite probable that losses in the former case were due to lack of regulation of the *p*H at which precipitation took place, and the above results demonstrate sufficiently conclusively that losses at the copper precipitation stage may be minimized by the use of glycerol and cupric sulphate, followed by decomposition with acetic acid. In this way the amount of inorganic salt introduced, and hence the loss on subsequent washing, is minimized. It should be pointed out that although the hemicelluloses of maize cobs investigated by Preece have been the subject of special comment in this paper, similar remarks probably apply to other hemicelluloses which have not yet been re-examined.

SUMMARY.

1. There is an optimum *p*H for precipitation of fraction A of a hemicellulose mixture.

2. Failure to precipitate at the optimum *p*H leads to a low yield of fraction A and the appearance of this fraction at later stages in the separation.

3. Improved yields of hemicellulose fractions may be obtained by precipitating with glycerol and cupric sulphate instead of Fehling's solution, and by subsequent decomposition with acetic acid instead of hydrochloric acid.

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CCCII. STUDIES ON HEMICELLULOSES.

VI. THE HEMICELLULOSES OF THE FLOWER OF THE HOP (*HUMULUS LUPULUS*).

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The study of the carbohydrate constituents of the hop flower constitutes an almost entirely neglected chapter in plant biochemistry. On the other hand, the chemistry of the antiseptic principles present in the hop has been the object of intensive research, chiefly owing to the known technical importance of these substances in the brewing processes. Nevertheless, a study of the complex carbohydrates of the hop should be of interest from both academic and technical viewpoints, since on the one hand we believe that this is the first occasion on which the hemicelluloses of floral organs have been described and on the other the colloidal systems formed by such substances in solution may have technical importance. The papers of Fink *et al.* [1935–36] on pectin in hops indicate a growing interest in the nature of this substance and its possible role in brewing technique.

It is proposed to describe studies on the nature of pectin in hops in a subsequent communication, whilst in this paper the preparation and properties of the hemicelluloses will be discussed.

The methods employed in the preparation of the hemicelluloses were similar to those indicated in earlier papers in this series [Norris & Preece, 1930; Preece, 1930], with such modifications as were found necessary in dealing with this particular source and several improvements in technique which have been indicated in the previous communication [Angell & Norris, 1936]. In addition, since the introduction of more exact methods of calculation of analytical results [Norris & Resch, 1935; Angell *et al.* 1936] we believe that we are now in a position to determine the proximate constitution of our products with greater accuracy than hitherto.

A more elaborate treatment of the material prior to extraction of the hemicelluloses was necessary in the case of hops since the amount of substances soluble in organic solvents was greater than in other materials examined by us. Failure to remove such substances from the original material led to considerable difficulty in purifying the final products which were invariably contaminated. The hops in bulk were therefore extracted to exhaustion successively with 95 % ethyl alcohol, methyl alcohol and ether. Extraction with water and hot ammonium oxalate solution followed, whereby all pectin was removed. The removal of lignin presents a considerable difficulty which Norris & Preece [1930] sought to overcome by extraction of the material with a 1 % solution of NaOH in 50 % alcohol. It was considered that while this reagent would remove a large proportion of the lignin, any tendency for the hemicellulose also to pass into solution would be checked by the presence of the alcohol. In a quantitative study Norman [1935] criticized this method on the ground that, in fact, hemicellulose was extracted and lost at this stage. We have carefully examined the material before and after extraction, and the product obtained by the extraction, in the cases of hops and of maize cobs, and in these two cases at least have found no appreciable loss of furfuraldehyde-yielding substances.

The hemicelluloses were extracted as usual with 4 % NaOH and fractionation was carried out with modifications of the method suggested by Norris & Preece [1930]. The first fraction, A, was most completely precipitated by acetic acid at *p*H 3, a more acid value than that for maize cob hemicelluloses [Angell & Norris, 1936]. The precipitation of fraction B with half a volume of acetone did not occur until the solution was further acidified to *p*H 2. On the other hand, fraction C was not precipitated at all in very acid solutions—*p*H lower than 4—but was precipitable by acetone in neutral or slightly acid solutions, with a feeble optimum at *p*H 5·5. Thus it would seem, as we have indicated in the previous communication, that the optimum *p*H of precipitation of the hemicellulose is a characteristic of each type.

Further fractionation was effected through the copper gels using cupric sulphate and glycerol for precipitation and acetic acid for decomposition [Angell & Norris, 1936]. In this way four fractions were obtained, namely A_1, B_1, B_2 and C_2, of which A_1 was by far the largest. The yields of B_1 and C_2 were so small as to preclude complete analysis.

Fractions A_1, B_2 and C_2 showed no striking differences in composition, since they all contained anhydroxylose, anhydroglucose, glycuronic anhydride and methoxyl groups. Quantitative examination revealed, however, that the proportions of each constituent varied very markedly from fraction to fraction. It is still not possible to say that any one fraction represents a single chemical entity, but the widely differing analyses show that some separation has been effected.

The total amount of hemicellulose is small, being approximately 3 %, but it will be seen from the above that the hemicelluloses conform to general type, consisting of pentosan, hexosan and uronic anhydride. It would appear therefore that no matter where the site may be, in the floral organs as in this case, or in the seed coats, stems etc. of a number of plants which have already been examined, the general constitution of the hemicelluloses is closely similar.

EXPERIMENTAL.

Analytical methods.

The following is a résumé of the methods employed throughout.

Moisture: by loss in weight on drying to constant weight in an electric oven at 100°.

Ash: by heating to a white ash of constant weight at dull red heat.

Protein: by standard Kjeldahl process; protein calculated as Kjeldahl nitrogen × 6·25.

Alcohol- and ether-soluble: by exhaustive extraction with each solvent in turn in a Soxhlet apparatus.

Cross and Bevan cellulose: by the usual modified procedure. The C. and B. cellulose yielded furfuraldehyde which was calculated to xylan by the equation $X=0{\cdot}7673P+0{\cdot}0069$ [Angell *et al.* 1936]. "True" cellulose was given by difference between C. and B. cellulose and xylan.

Lignin: by method of Schwalbe [1925] involving treatment with a mixture of 72 % sulphuric acid and 18 % hydrochloric acid.

Uronic anhydride: by the apparatus of Dickson *et al.* [1930], modified by Norris & Resch [1935].

Furfuraldehydephloroglucide: by the method of Kröber [1901] using a modified apparatus. As an approximate indication of pentosan and urone content in the hops at various stages the "yield of furfuraldehyde" is given by use of the equation $F=0{\cdot}5198P+0{\cdot}0020$. In mixtures containing xylose, glycuronic anhydride and glucose, anhydroxylose, X, is obtained from the equation

$$X=0{\cdot}0060+0{\cdot}7859P-1{\cdot}146C,$$

where P = phloroglucide and C = carbon dioxide from urone. Glucose is given approximately by difference [Angell *et al.* 1936].

Pretreatment of hops.

The hemicelluloses were prepared from the fresh unsulphured flowers of the hop, variety Fuggles, grown in Worcestershire and picked in September 1934.

Analysis: The fresh hops contained 76·6 % moisture and the dry hops gave the following analysis:

	%
Ash	8·5
Protein	21·3
Alcohol-ether-soluble	30·6
C. and B. cellulose	15·3
Xylan	2·3
True cellulose	13·0
Lignin	22·8
Furfuraldehyde	6·4

The total hemicellulose present was determined by the method of Preece [1931] and found, on an ash-free basis, to be 3·4 %. An attempt to determine the amounts of each fraction [Buston, 1934] proved unsuccessful owing to the extreme difficulty in separating small amounts of colloidal precipitate.

Extraction with organic solvents. About 6500 g. of hops were dried in air at 50° for 5 days, then ground in a Christie and Norris laboratory mill and immersed immediately in 80 % alcohol in order to minimize enzyme action on rupture of the cells. The net dry weight of hops used was 1510 g.

The ground hops were filtered from the 80 % alcohol, placed in a calico bag and extracted with boiling 95 % alcohol for 10–12 hours on each of 9 days. The ethyl alcohol was then expressed and a similar treatment with methyl alcohol for 8 days followed. After removal of methyl alcohol, the hops were extracted twice with ether under reflux for 3 hours, washed with ether, dried and weighed. The weight after this treatment was 950 g. corresponding to a loss of 37 % on the original dry weight. The furfuraldehyde yield of the extracted hops was 9·8 %, equivalent to 6·2 % on the original dry hops. Since the latter gave a furfuraldehyde yield of 6·4 % it is evident that no appreciable loss of pentosan or uronic anhydride had occurred by reason of the extraction.

Extraction of pectin. Since hop pectin was required for the purpose of another investigation the opportunity was taken to prepare pectin by different treatments and in this way the hops were completely exhausted of pectin. They were extracted successively by water: (i) at room temperature, (ii) at 50°, (iii) at 90°, (iv) under pressure at 125°; and finally three times with hot 0·5 % ammonium oxalate. The residue was thoroughly washed and pressed.

Extraction of lignin. The hops were then extracted twice with boiling 1 % NaOH in 50 % alcohol as suggested by Norris & Preece [1930]. A third extraction with 50 % alcohol only was carried out in order to facilitate removal of the NaOH, after which the hops were filtered off and pressed.

The alcoholic NaOH extract was examined in order to detect any possible loss of hemicellulose at this stage as suggested by Norman [1935]. The solution was acidified with glacial acetic acid to pH 3·5, a copious brown precipitate being formed. This was reprecipitated from 1 % NaOH by acetic acid, washed and dried in graded strengths of alcohol. It was then extracted in a Soxhlet extractor with methyl alcohol, which removed a quantity of brown-coloured material, then with absolute alcohol and ether. After drying *in vacuo* the powder weighed 17·2 g., equivalent to 1·15 % of the hops. The product contained 68 % of lignin and 70 % was unaffected by hydrolysis for 12 hours with 0·5N sulphuric acid. From the present viewpoint, however, the most important fact is its failure to yield any furfuraldehyde at all. This shows conclusively that hemicellulose—in particular fraction A, which would be precipitated under the conditions imposed—was not dissolved out by the reagent. A similar result was obtained in the case of maize cobs which were specially re-examined in view of Norman's results.

Extraction of hemicelluloses.

The residue after lignin extraction was allowed to stand overnight with a large volume of cold 4 % NaOH, the extract then being filtered off through muslin. This procedure was repeated six times, until the last extracts contained only a negligible quantity of material precipitable by acetone.

Precipitation of hemicellulose A. Preliminary experiments, similar to those described in the previous communication [Angell & Norris, 1936], had shown that the most complete precipitation of fraction A occurred at pH 3. In order to bring the alkaline solution to this acidity it was necessary to use hydrochloric in addition to acetic acid. Accordingly a mixture of these acids was added to the united NaOH extracts until this acidity was reached, the precipitate being allowed to settle out and then separated at the centrifuge or by filtration. The precipitate was redissolved and reprecipitated several times and finally dried in graded strengths of alcohol and *in vacuo*.

For separation through the copper gel the following procedure was adopted, again as the result of preliminary experiments. The fraction is dissolved in 4 % NaOH and to the solution is added one-twentieth volume of glycerol, the whole being thoroughly stirred. Stirring is continued whilst one-tenth volume of molar cupric sulphate is added. If the gel formed is bulky it may be filtered through muslin and pressed out; if small in quantity it may be filtered off on a hardened paper. The gel is then stirred into water and decomposed with acetic acid; from the solution it is precipitated by acetone, reprecipitated and dried as usual.

The copper solution after separation of the gel is also treated with acetone for separation of the other fraction if present. In this instance, no A_2 fraction was obtained, the whole of the original precipitate coming down as copper gel of fraction A_1.

Precipitation of hemicellulose B. The filtrate after separation of the first fraction A was brought to pH 2 and one-half volume of acetone added. The precipitate formed was fractionated, as previously described, through the copper gel, and in this case two fractions were obtained, B_1 and B_2.

Precipitation of hemicellulose C. The filtrate from hemicellulose B was adjusted to pH 5·5 and excess acetone added whereby a final precipitate was obtained which on copper treatment was found to consist solely of fraction C_2.

Composition of the hemicelluloses.

The final products varied in colour from white to yellowish brown; they contained varying amounts of ash and lignin, which could not be completely removed. The hemicelluloses were non-reducing and gave no colorations with iodine. Fractions A_1 and B_1 were sparingly soluble in boiling water, but soluble in alkali; on the other hand B_2 and C_2 were readily soluble in cold water. The yields of each fraction and their analyses are given in Table I.

Table I.

Fraction	Ash %	Lignin %	Yield g.	Yield %	Furfuraldehyde %	Carbon dioxide %	Methoxyl %
			On a lignin- and ash-free basis				
A_1	0·85	2·04	24·1	1·6	50·5	3·12	1·43
B_1	8·9	—	0·6	0·04	24·5*	3·83*	—
B_2	12·7	12·0	4·0	0·26	39·6	3·53	4·55
C_2	12·7	10·4	1·5	0·10	19·6	9·70	1·37

* Ash-free basis only.

Hydrolysis of A_1 and B_2 with dilute sulphuric acid and subsequent identification of the sugars formed showed that in each case xylose and glucose were present, whilst the Heidelberger & Goebel [1927] reaction indicated glycuronic acid. Anhydroxylose, X, in the products was calculated from the yields of phloroglucide, P, and carbon dioxide, C, using the equation

$$X = 0{\cdot}0060 + 0{\cdot}7859P - 1{\cdot}146C.$$

Anhydroglucose was then obtained by difference. The limits of accuracy of this procedure have already been discussed [Angell *et al.* 1936].

The yields of B_1 and C_2 were so small that it was not possible to complete a qualitative examination; in the latter case it is possible to calculate the proximate composition from the quantitative results, on the assumption that the hydrolysis products are the same as for A_1 and B_2. The following table shows the percentage composition of the three major fractions.

	Fractions A_1	Fractions B_2	Fractions C_2
Anhydroxylose	76·1	58·2	20·9
Glycuronic anhydride	12·5	14·1	38·8
Methoxyl	1·4	4·5	1·4
Anhydroglucose (by diff.)	10·0	23·2	38·9

Fraction B_1 was so small in amount as to suggest that it might possibly represent a small proportion of fraction A which had escaped precipitation. In the absence of data on the lignin content of B_1 it is impossible to establish its composition with as much accuracy as in the other cases, but the figures available show that it must consist of 35–39 % of anhydroxylose, 15–17 % of uronic anhydride and about 47 % of anhydroglucose. These values are sufficiently different from any of the others in the above table to show plainly that this fraction has a separate existence.

DISCUSSION.

The composition of the hemicelluloses in every case shows that anhydroxylose is the only pentose present and, as might be anticipated, this is accompanied by glycuronic anhydride and anhydroglucose. It is somewhat surprising that the hemicelluloses present in the floral organs should show such close similarity to those obtained from various woods. Thus O'Dwyer found large amounts of xylan together with glycuronic anhydride and methoxyl groups in hemicelluloses of beech [1926] and oak [1934], whilst those of mesquite wood were similar [Sands & Gary, 1933]. Nevertheless, it is significant that the dried hops contained some 22% of lignin, and the products fall into line with Buston's proposed classification [1935]. Thus, according to Buston, non-lignified tissues may be expected to yield pectin and hemicelluloses of the galactose, arabinose and galacturonic acid series, whilst lignified tissues yield little or no pectin and hemicelluloses of the xylose, glucose and glycuronic acid series. It is evident then that hop flower hemicelluloses will fall into the latter class.

We have stressed the impossibility of establishing the individual chemical nature of any one of the hemicellulose fractions and would also point out the danger in attempting, at this stage, any correlation between chemical constitution and physical characteristics. Thus, Buston [1935] has attempted to show that insoluble "A" hemicelluloses consist largely of either pentosan or hexosan units, whilst the soluble "B" and "C" types contain large proportions of uronic anhydride. That this is not universally the case is indicated by fractions A_1 and B_2 in hops: the marked difference in physical properties in this case is to be correlated merely with an alteration in the pentosan/hexosan ratio, the proportion of uronic anhydride remaining substantially unaltered.

Finally, it may be mentioned at this stage that the whole of the work described here was preceded by preliminary operations on hops of the 1933 season. Such work need not be detailed here, since the methods adopted for the 1934 crop were in every way superior. Some evidence, however, was obtained which appears to indicate that the relative amounts and proximate composition of the various fractions of hemicelluloses may be subject to seasonal and other variations.

SUMMARY.

1. The hemicelluloses of the flowers of the hop have been prepared and their nature investigated.

2. The importance of the *p*H of the solutions for precipitating the various fractions is stressed.

3. The fractionation by means of copper solutions is carried out with glycerol-cupric sulphate mixture.

4. The largest fractions are A_1 and B_2, whilst C_2 and particularly B_1 are small.

5. All fractions consist of anhydroxylose, anhydroglucose and glycuronic anhydride in different proportions and hence the hop hemicelluloses belong to the xylan class usually found in lignified tissues.

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CCCIII. LIVER XANTHINE OXIDASE.

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IN the past the Schardinger enzyme-xanthine oxidase system has received considerable attention, but investigations have been mainly confined to the preparation and properties of the enzyme as found in milk. Liver of most Mammalia is a potent source of this enzyme, but apart from the pioneer work of Battelli & Stern [1910], and Parnas [1910], the only detailed investigation of this source is that of Wieland & Frage [1929].

Milk is of course a much better starting material for the preparation of the enzyme, as not only is it almost free from accompanying substrates, but it is not a rich storehouse of enzymes like liver. Despite this it seemed of interest to study the properties of liver enzyme preparations, if only to compare them with those from milk.

The earlier work of Wieland and Frage, of which this communication forms an extension, was carried out with horse liver. The difficulty of obtaining supplies soon led to its abandonment in favour of ox liver. A further point in favour of ox liver is that, unlike horse liver, it is free from uricase [Rô, 1931], a fact later confirmed in this investigation. This makes the interpretation of figures obtained for the oxidation of purine bases to uric acid much easier as there is no further oxidative breakdown to allantoin to consider.

I. RESPIRATION OF MINCED LIVER AND ITS AQUEOUS EXTRACTS.

In studying the respiration of minced ox liver it became evident that owing to factors unknown at the time, certain of the results of Wieland & Frage could no longer be considered correct. These authors stated that the rate of oxygen uptake of minced liver suspended in phosphate buffer slowed down considerably after half an hour, whilst the corresponding development of CO_2 came to a standstill at the same time. In the experiments from which these conclusions were drawn a 40 % solution of KOH was used to absorb CO_2 developed. This method of CO_2 absorption has been shown by Dixon & Elliott [1930] to be very inefficient, and when a small roll of filter paper soaked in 7 % KOH is used instead, quite different results are obtained. As is shown in Table I both oxygen uptake and CO_2 output continue quite regularly, the rates fall off slowly with time, but there is no sudden decrease or cessation.

The CO_2 output is calculated by difference from the manometric readings obtained when no KOH is used to absorb the CO_2 produced.

This does not give a strictly accurate measure of the CO_2 output as both the phosphate buffer and the protein present will retain a certain amount of CO_2. The R.Q. 0·37 as calculated from the above data is therefore low. Figures are not yet available for the R.Q. of ox liver slices, but Gemmill & Holmes [1935] record 0·79 for rat liver slices. It therefore seems likely that mincing leads to a

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Table I.

Time in min.	O_2 uptake µl./g.	CO_2 output µl./g.
15	92	44
30	178	66
45	260	92
60	322	116
90	434	160
120	536	206

reduction in the R.Q. of ox liver, a conclusion which it is hoped to confirm at a later date. It is a well-known fact that mincing leads to inactivation of several enzymes in tissues. As xanthine oxidase is unaffected by such treatment, mincing frees the ultimate preparation from some of the enzymes likely to be present.

Acceleration of respiration by xanthine.

An aqueous extract of minced liver (1 ml. per g.), like the 'brei' itself, has quite a considerable self-respiration. If xanthine be added to a solution containing 1 ml. of such an extract buffered at *p*H 7·6, the respiration may or may not undergo an initial acceleration. It may take an hour or more for xanthine to exert an accelerating effect, but ultimately the extra oxygen uptake will amount to that required for the complete oxidation of xanthine to uric acid.

Similar experiments by Morgan *et al.* [1922] showed that with rat liver the oxygen uptakes were in accord with the complete conversion of xanthine into allantoin. As rat liver is rich in uricase and ox liver contains none the discrepancy is only apparent. The aqueous extracts are kept sterile by toluene; otherwise, as shown by Truszkowski [1930], bacterial contamination may lead to uricolytic action. Toluene does not seem to affect xanthine oxidase in any way.

Table II shows the varying effects of the addition of $M/100$ or $M/80$ xanthine on the rate of respiration of an aqueous extract of liver.

Table II.

Q_{O_2}	$Q^X_{O_2}$	Q_{O_2}	$Q^X_{O_2}$
104	129	70	152
100	149	82	154
62	172	164	164
127	131		

Q_{O_2} represents the amount of oxygen taken up by 1 ml. of an aqueous liver extract in 1 hour, whilst $Q^X_{O_2}$ represents the amount taken up when xanthine is also present. Uptakes are recorded in µl.

While $Q^X_{O_2}$ only varies some 15 % on either side of a mean, Q_{O_2} is subject to wide variations. $Q^X_{O_2}$ may be equal to Q_{O_2}, or it may be three times as great. The approximate constancy of $Q^X_{O_2}$ indicates that the activity of xanthine oxidase does not vary much from one liver to another, but variations in Q_{O_2} indicate a variable purine content.

However suboptimum concentrations of purine bases are so low that for a complete explanation of the respiration rate obtained a continuous formation of purine base by nucleosidase action must be assumed. The purine base is oxidized as soon as it is set free so that its rate of oxidation is governed by its rate of formation.

Precisely the same process takes place in the milk enzyme. According to Dixon & Lemberg [1934] the slow hydrolysis of inosine by xanthine oxidase preparations can be followed manometrically, as the hypoxanthine is oxidized to uric acid as soon as it is formed.

The above results are not inconsistent with the view that the bulk of the respiration of minced liver is due to the oxidation of xanthine. Although positive proof of this cannot be advanced there is evidence which fits in with this view. If the bulk of the respiration is due to xanthine oxidase it should be but little affected by cyanide for, as will be shown later, the enzyme is only partially inhibited at high cyanide concentrations. In agreement with earlier results of Dixon & Elliott [1929] it has been found that this is indeed the case, inhibition amounting to less than 20% with $M/1000$ HCN. The extent of cyanide inhibition is thus in harmony with, although it does not prove, the theory advanced.

If we consider the non-cyanide-sensitive part of the respiration to be due to purine base oxidation it should be possible to calculate the purine base content of the liver. In one case, typical of several, the self-respiration of 1 ml. of an aqueous liver extract in the presence of $M/1000$ HCN amounted to 173 μl. after 6 hours' shaking, by which time the reaction had come almost to a standstill. If the whole of this oxygen uptake were due to xanthine oxidation the purine base content of the extract would be 2·35 mg./ml. while if due to hypoxanthine oxidation it would be 1·05 mg./ml. Thus the soluble purine base content of ox liver if the above views are correct should be between 105 and 235 mg. per 100 ml., which is consistent with the value 201 mg. per 100 ml. found by Fellenberg [1918].

Mincing the tissue does not necessarily destroy enzymic systems, but those systems requiring a coenzyme will have their activities largely reduced by the dilution effect of the dispersion of the coenzyme. Other enzymes such as the succinic dehydrogenase may make but little contribution to the respiration rate owing to the low substrate concentration associated with the enzyme. That this applies in the case of succinic dehydrogenase is shown by the very great acceleration in the rate of oxygen uptake when succinate is added to minced liver.

Effect of acetaldehyde on the respiration rate.

According to Wieland & Frage the addition of acetaldehyde has an accelerating influence on both the oxygen uptake and the CO_2 output of respiring liver extracts. This acceleration in oxygen uptake is however only apparent, as the increase is exactly equal to the rate of autoxidation of acetaldehyde when alkali is present to absorb CO_2.

Thus after 1 hour's shaking the oxygen uptake of 218 μl. shown by 0·25 g. of minced liver is increased to 317 by $M/25$ acetaldehyde. This concentration of acetaldehyde under the same conditions produces an oxygen uptake of 102 μl. without any enzyme being present.

According to Andersson [1935] the dehydrogenation of acetaldehyde by the Schardinger enzyme is strongly inhibited by several purine derivatives such as adenosine, muscle adenylic acid and adenylpyrophosphate as well as by xanthine itself. The presence in liver of these substances accounts for the failure of the enzyme to bring about the oxidation of its substrate. This conclusion implies that the oxidation of acetaldehyde by the liver enzyme has no physiological significance. In milk, which is largely free from purine derivatives, added aldehydes are oxidized quite normally.

When no steps are taken to absorb the CO_2 produced one would expect that the addition of acetaldehyde would have no effect on the manometric readings, but actually the readings were distinctly higher. As oxygen uptake is unaffected, CO_2 output must therefore be inhibited, probably through an inhibition by the aldehyde of enzymic processes leading to CO_2 formation. In microrespiration studies of the oxidation of aldehyde, CO_2 must therefore be absorbed even although it is not a product of oxidation of the substrate, unless one can prove quite definitely that no CO_2 is produced in the system at all.

II. PREPARATION OF XANTHINE OXIDASE FROM LIVER.

In the earlier work of Wieland & Frage [1929] an enzyme preparation is described obtained by the precipitation with acetone of a toluene-water extract of finely minced liver. If this precipitate is centrifuged down, exhaustively washed with acetone and given one final washing with ether (dried and distilled over sodium) an enzyme preparation is obtained in the form of a very finely divided powder (Prep. A).

It is insoluble in water, but its suspension functions as quite an active catalyst. It still possesses an appreciable self-respiration but the accelerations in the rate of oxygen uptake on addition of xanthine and aldehyde are quite marked.

Its insolubility makes it impossible to concentrate the enzyme by dissolving and reprecipitating. Fractional precipitation with acetone led to no definite concentration of the enzyme in any one particular fraction. Further, attempts to deplete the liver mince of its substrates, either by dialysis or impoverishment (shaking with oxygen for several hours) were not sufficiently successful to make such preliminary treatment before acetone precipitation of any great advantage.

In manometric experiments 0·2 or 0·25 g. proved a convenient amount to handle. With the Warburg technique this amount was suspended in 5 ml. $M/15$ phosphate buffer, pH 7·6, and the volume adjusted with distilled water so that after addition of substrate the total volume was 8 ml. In the experiments with the Barcroft differential manometer the total volume was 3 ml.

Of many substrates tried only purine bases and aldehydes accelerated the rate of oxygen uptake. There was no acceleration on the addition of succinic acid or alcohol despite the high activity in liver of their corresponding dehydrogenases.

In an endeavour to make the enzyme more water-soluble the minced liver was subjected to digestion with takadiastase and with trypsin, but both methods brought about a complete destruction of the xanthine oxidase.

The dehydration of minced liver with acetone has led, in the hands of Bernheim [1928, 2] and Harrison [1931, 1, 2], to a method by which several dehydrogenases could be extracted from the acetone-liver. An aqueous extract of acetone-liver may itself be used as an enzyme source but a solid preparation may be obtained by adding slowly, with stirring, an equal volume of a saturated solution of ammonium sulphate. A high-speed centrifuge is required to separate the precipitate formed. It is roughly dried on a porous plate to remove as much ammonium sulphate solution as possible, after which drying is completed *in vacuo* over sulphuric acid (Prep. B).

This preparation is completely soluble in water or phosphate buffer, and 1 ml. of a 10 % solution in $M/15$ phosphate pH 7·6 is a convenient amount for manometric experiments.

Acetone-liver, like aqueous liver extracts, has a somewhat variable self-respiration but its oxygen uptake is always considerably accelerated by the addition of xanthine. Its phosphate extracts have a somewhat lower self-respiration and the final preparation is sometimes obtained completely free from self-respiration.

Oxidation of aldehydes.

These experiments were all carried out with Prep. A. If in manometric experiments on the oxidation of volatile aldehydes KOH is used to absorb any CO_2 that may be formed, there will be a rapid oxygen uptake due to a condensation reaction of the aldehyde at the surface of the KOH. This, unless eliminated or corrected for, will lead to very inaccurate results, and failure to realize this fact invalidates much of the previous work on the enzymic oxidation of these aldehydes.

In this particular work CO_2 must be absorbed, so the effect was corrected for by subtracting values for the non-enzymic autoxidation from the values recorded. This procedure seems justifiable as the two reactions leading to an oxygen uptake occur in different phases and should not interfere with one another.

Table III.

µl. O_2

Time min.	Acetaldehyde. *M*/10	*M*/25	*M*/50
10	60	26	13
20	105	45	20
30	—	64	28
40	176	73	34
80	289	123	—

Time min.	Propaldehyde. *M*/10	*M*/20
5	90	40
10	142	66
15	188	91
20	226	111
30	298	151
40	364	191
50	420	227
60	472	261

The figures quoted above are average values from often as many as ten determinations; individually the uptakes vary considerably. There is an approximate dependence of the rate of autoxidation on the aldehyde concentration.

In Figs. 1 and 2 are plotted the results obtained for the enzymic oxidations of acetaldehyde and propaldehyde, corrected in this way for autoxidation.

The rate of oxidation of acetaldehyde is independent of substrate concentration so long as this concentration is greater than *M*/50. In each of several such experiments the values for *M*/100 acetaldehyde were consistently lower, and for *M*/250 acetaldehyde lower still. The figures obtained were not precise enough for an exact evaluation of the Michaelis constant, but it is probably of the order 0·005 *M*.

Propaldehyde undergoes autoxidation much more rapidly so that a larger proportion of the recorded uptake is due to this factor. Despite the greater error introduced there seems little reason to doubt that the initial rates of oxidation of propaldehyde are the same while the concentration ranges from *M*/100 to *M*/10. In the later stages of the reaction this agreement is not maintained, and after an hour's shaking the uptake with *M*/100 aldehyde is double that with *M*/10. In the latter case reaction ceases after 30 min.

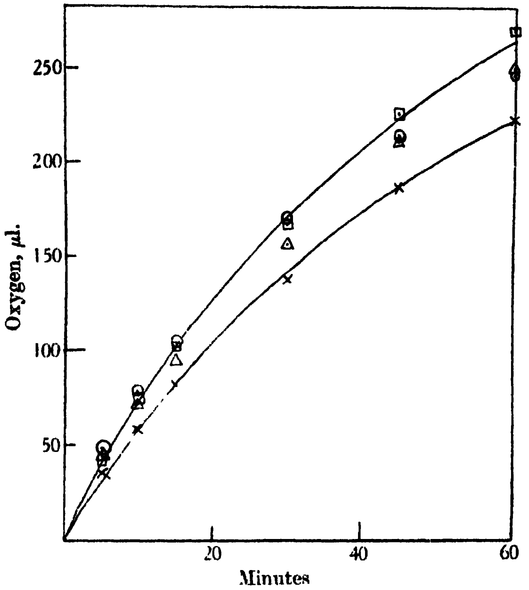

Fig. 1. ⊙—⊙ *M*/10 acetaldehyde. △—△ *M*/25 acetaldehyde. ⊡—⊡ *M*/50 acetaldehyde. ×—× *M*/100 acetaldehyde.

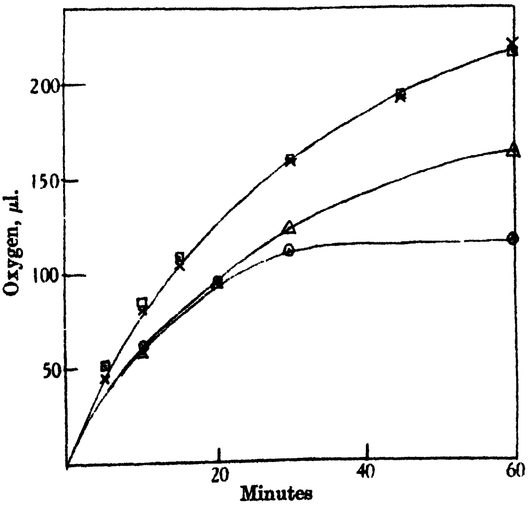

Fig. 2. ⊙—⊙ *M*/10 propaldehyde. △—△ *M*/25 propaldehyde. ⊡—⊡ *M*/50 propaldehyde. ×—× *M*/100 propaldehyde.

With acetaldehyde the uptake curve is not linear, and when we consider that the enzyme is protected against H_2O_2 inhibition by catalase and is unaffected by acetate and by mechanical shaking, we must conclude that acetaldehyde inhibits its own dehydrogenase. Propaldehyde appears to have an even higher degree of toxicity and here the slowing up of the reaction rate is sufficiently marked for the greater effect of higher substrate concentrations to be evident.

If these conclusions are to be accepted incubation of xanthine oxidase with *M*/10 propaldehyde should cause complete destruction of the enzyme. To test this advantage was taken of the insolubility of Prep. A. After incubation at 37° for 1 hour the enzyme was centrifuged down, washed to remove products of reaction and resuspended in phosphate buffer. After this treatment it was found to be completely inactive. The centrifugate, on the other hand, could still function as a substrate for fresh enzyme so that it can be concluded that no toxic products are formed as a result of the reaction and that it is the substrate itself that is the inhibitor.

It is interesting to recall that Battelli & Stern [1913] showed that propionaldehyde exerted a greater toxic effect than acetaldehyde on succinic dehydrogenase, and that Bernheim [1928, 1] showed that the aldehyde dehydrogenase of potato was completely destroyed by incubation with acetaldehyde.

Oxidation of xanthine.

Using Prep. B, one completely free from self-respiration, the theoretical curve for the complete oxidation of xanthine to uric acid was realized, as shown in Fig. 3.

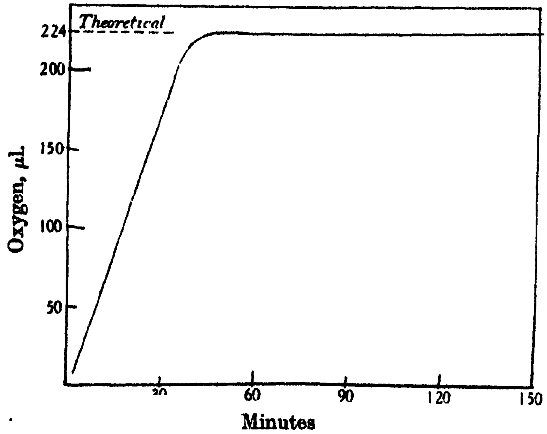

Fig. 3.

The rate was linear until oxidation was nearly complete and, after the calculated quantity of oxygen had been taken up (2 ml. *M*/100 xanthine requires 224 µl. O_2), the reaction came to a standstill. The curve is identical with that obtained by Dixon [1925] when milk xanthine oxidase was mixed with a sufficiency of catalase. As is well known, liver is rich in catalase. 0·02 *M* H_2O_2 is instantaneously decomposed by a phosphate extract of acetone-liver and the rate is only measurable after considerable dilution.

With this high catalase concentration the enzyme is completely protected against the toxic effect of H_2O_2. The uptake curves are therefore linear and as the H_2O_2 is completely decomposed the uptakes for complete oxidation to uric acid are realized exactly.

The enzymic oxidation of xanthine by liver xanthine oxidase is perfectly normal. In Figs. 4 and 5 are plotted the results obtained by varying enzyme and substrate concentration.

The initial rate of oxidation is proportional to the enzyme concentration. A five-fold increase in the substrate concentration has no effect on the rate and the two curves only separate when the oxidation of $M/400$ xanthine approaches completion. Beyond this point the curve obtained with $M/80$ xanthine still follows the same straight line. The persistence of the straight line up to the point where oxidation is almost complete indicates that the rate is independent of substrate concentration down to very high dilutions of the order of $M/4000$. This indicates a low K_m of the same order as that found by Dixon & Thurlow [1924] and by Booth [1935]. Apparently the purine bases do not inhibit their own oxidation when oxygen, and not methylene blue, is the hydrogen acceptor.

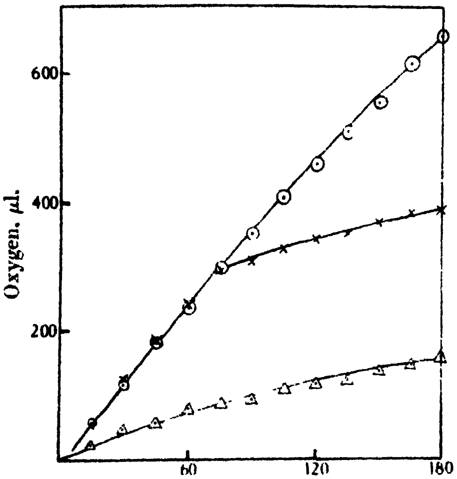

Fig. 4.

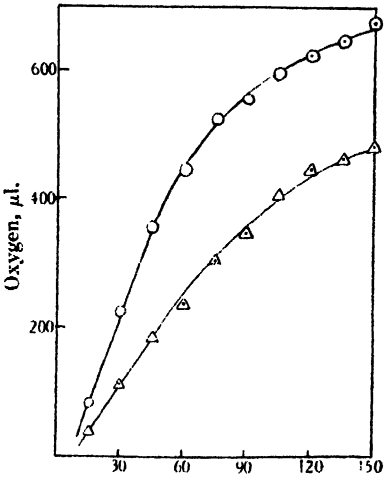

Fig. 5.

Fig. 4. ⊙—⊙ $M/80$ xanthine. ×—× $M/400$ xanthine. △—△ Self-respiration.

Fig. 5. ⊙—⊙ 0·50 g. Prep. A. △—△ 0·25 g. Prep. A. $M/200$ xanthine.

The straight line uptake curves do not pass through the origin but cut the time axis at a point corresponding to a time interval of 5 to 10 min. Dixon [1925] noticed a similar effect, for when strong catalase solutions were added to milk preparations, there was always an initial induction period.

Cyanide inhibition.

With both Prep. A and B cyanide inhibition of the oxidation of xanthine is quite marked although it does not approach the order of inhibition observed with the succinic dehydrogenase. The effect of varying concentrations of cyanide on the rate of oxidation of xanthine is shown in Fig. 6.

30 % of the activity, as measured by initial rate of reaction, is retained in the presence of $M/100$ HCN. Such a concentration would cause complete inhibition of O_2 uptake of succinic oxidase, while according to Dixon & Thurlow [1925] *milk xanthine oxidase is unaffected by cyanide at this concentration. The essential difference between Dixon's milk preparations and liver xanthine*

oxidase is in the high catalase content of the latter. A closer inspection of the inhibition by high cyanide concentrations shows that the oxidation comes almost to a standstill with less than a half of the xanthine oxidized. This is so reminiscent of the oxidation of xanthine by catalase-free xanthine oxidase which Dixon [1925] showed to be brought to a standstill by the accumulation of H_2O_2, that it was concluded that similar conditions operated here through the complete inhibition of the catalase by HCN.

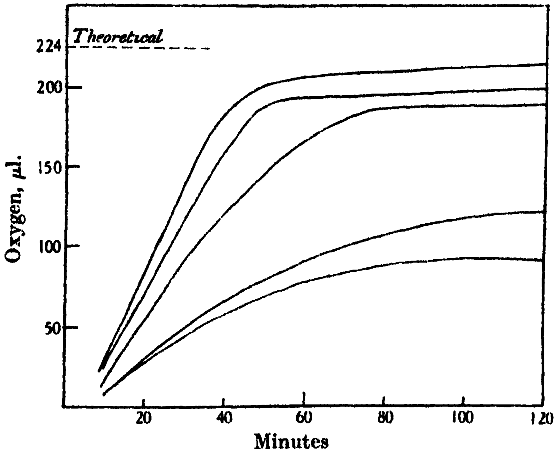

Fig. 6. I, Oxidation $M/400$ xanthine. II, Oxidation + $M/2500$ HCN. III, Oxidation + $M/500$ HCN. IV, Oxidation + $M/200$ HCN. V, Oxidation + $M/100$ HCN.

However, it has recently been shown by Dixon & Keilin [1936] that incubation of xanthine oxidase with HCN in the absence of substrates caused a progressive inactivation. As, in the above experiments, HCN was mixed with the enzyme and the substrate added after the attainment of temperature equilibrium, the above results might be due to this same type of inhibition. To test this possibility the experiments were repeated, and the effect of adding the cyanide together with the substrate, after the attainment of temperature equilibrium, was compared with that observed when enzyme and inhibitor were mixed initially. The extent of inhibition is slightly, although significantly, less when substrate and cyanide are added at the same time as shown in Fig. 7, but the results leave no doubt that the Dixon type of inhibition accounts for but a fraction of the total inhibition observed. If the Dixon effect were wholly responsible, there would be no inhibition at all when the cyanide was added at the same time as the substrate. It can therefore be concluded that the cyanide-sensitivity of liver xanthine oxidase is due mainly to the inhibition of its catalase.

Aerobic mixed substrate tests for xanthine oxidase have been carried out by Sen [1931] using hypoxanthine and the non-volatile aldehydes, piperonal and vanillin. It seemed of interest to see whether volatile aldehydes showed a similar competitive inhibition, making, of course, appropriate corrections for autoxidation. The substrates chosen were $M/50$ propaldehyde and $M/200$ xanthine both of which are in sufficiently high concentration to saturate the enzyme. The results obtained are plotted in Fig. 8.

No conclusions can be drawn from these results as to the single or dual nature of the enzyme. Saturation of the enzyme with xanthine appears to inhibit the oxidation of the aldehyde as initially the rate with the mixed substrate is exactly the same as with xanthine alone. It has also been shown by Andersson [1935] that xanthine inhibits the oxidation of aldehydes. The falling off in the rate with the mixed substrate can be ascribed to the toxic effect of the propaldehyde.

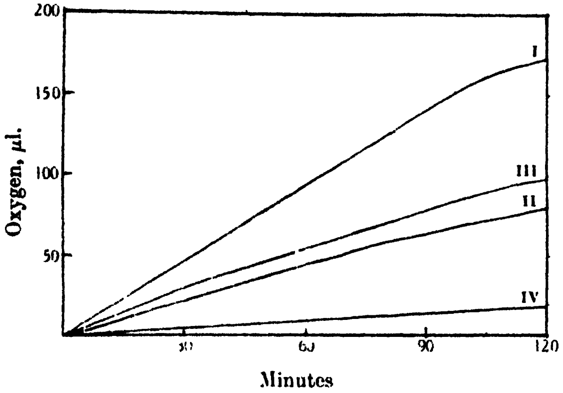

Fig. 7. I, *M*/100 xanthine. II, Inhibition by *M*/100 HCN. III, Inhibition by *M*/100 HCN added with substrate. IV, Self-respiration.

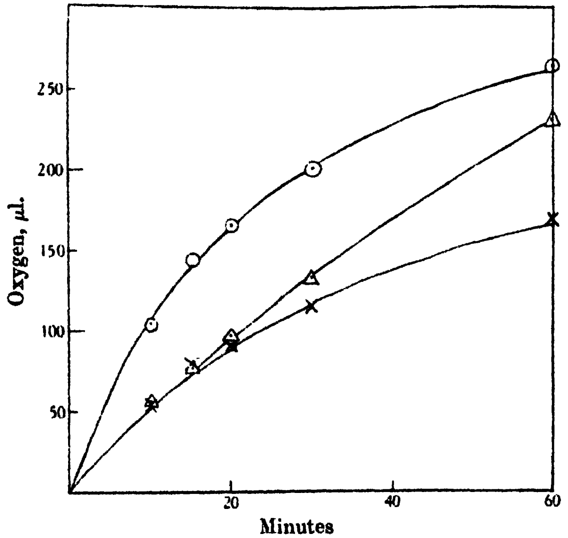

Fig. 8. ⊙—⊙ *M*/50 propaldehyde. △—△ *M*/200 xanthine. ×—× Mixed substrate.

SUMMARY.

1. In contradistinction to earlier work by Wieland & Frage the respiration of minced liver proceeds normally without any sudden slowing down.

2. The observed rate of self-respiration of minced liver may be ascribed mainly to purine-base oxidation, and this rate is controlled by the rate of formation of purine base by nucleosidase action.

3. Acetaldehyde has no accelerating influence on the oxygen uptake of minced liver and inhibits its CO_2 output.

4. The kinetics of aldehyde oxidation can be measured manometrically if a correction is made for the non-enzymic autoxidation of the aldehydes on the surface of the KOH. For acetaldehyde K_m is probably of the order of 0·005 M. Propaldehyde and, to a less extent, acetaldehyde destroy the enzyme which catalyses their oxidation.

5. Liver xanthine oxidase contains sufficient catalase to protect it against H_2O_2.

6. Cyanide inhibits xanthine oxidation indirectly by poisoning the catalase.

7. Aerobically a mixture of xanthine and propaldehyde is oxidized at the same rate as xanthine alone.

I wish to express my gratitude to Prof. Wieland, who suggested this problem to me, and to Prof. Peters for much helpful criticism and advice during the course of this work. My thanks are also due to the Department of Scientific and Industrial Research for a grant.

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CCCIV. THE DISTRIBUTION OF MANNAN IN SOME GYMNOSPERMS.

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Mannan has long been known to be widely distributed in woods, particularly in the softwoods or gymnosperms. Bertrand [1899] drew attention to the fact that whereas the hardwoods or angiosperms contain much xylan and little mannan, the softwoods contain more mannan than xylan and suggested that the mannan in a softwood occupies the same place as xylan in a hardwood. Schorger [1917] showed mannan to be present in 20 different species of softwoods in amounts up to 7·6%. Later it was realized that some of the mannan of softwoods is associated with the cellulosic fraction, and mannose was obtained from wood cellulose and wood pulps by hydrolysis [Lenze *et al.* 1920]. Heuser & Dammel [1924] found that the mannan of spruce pulp passes into the β-cellulose fraction, and Hägglund & Klingstedt [1927] demonstrated the presence of mannan in specimens of wood cellulose by a consideration of the optical rotations of the cuprammonium solutions. Actual preparation of the mannan from sulphite pulp was achieved by Hess & Lüdtke [1928], who showed that its physical properties were similar to those of the mannan extracted from the ivory nut (*Phytelephas macrocarpa*).

The mannan associated with the cellulose of softwoods may be regarded as a cellulosan according to the nomenclature proposed by Hawley & Norman [1932], just as the xylan of hardwoods is so described. The xylan content of cellulose preparations is frequently determined in systems of analysis, so that a distinction may be drawn between "pentosans in cellulose" and "pentosans not in cellulose", but much less attention has been given to the mannan of softwoods. The purpose of the work described below therefore was to determine the distribution of the mannan in a few species of softwood and to examine the conditions of removal of the mannan associated with the wood cellulose.

Experimental.

I. *The determination of mannan.*

All methods of determination of mannan are based on the precipitation of mannosephenylhydrazone in the cold, and some modification of the procedure described by Bertrand [1899] is usually employed, such as that of Schorger [1917]. Briefly this consists of hydrolysing the wood by boiling for 3½ hours with 5% HCl, neutralizing the extract, concentrating and precipitating the mannose by the addition of phenylhydrazine and acetic acid. Alternative methods involving extraction of mannan with alkali prior to hydrolysis have been shown to be inaccurate owing to incomplete extraction. It is not on record, however, that the quantitative aspects of the procedure recommended by Schorger [1917] have been studied, and in view of the fact that it is frequently necessary to determine mannose in low concentration this point was examined.

It was first established that the mannose produced on hydrolysis is stable to the mineral acid employed. No apparent fall in reducing value occurred when pure mannose was boiled with 5 % HCl for periods up to 5 hours, the Shaffer-Somogyi micro-method being used.

The yield of hydrazone from different concentrations of mannose precipitated with (*a*) phenylhydrazine and acetic acid, and (*b*) phenylhydrazine hydrochloride and sodium acetate has been investigated, and the effect of the presence of sodium chloride examined. Some of the data from these experiments are given in Table I.

Table I. *Precipitation of hydrazone from different concentrations of mannose under varying conditions.*

Phenylhydrazine : sugar = 1 : 10. Stand at room temperature.

Conc. of mannose %	Sodium chloride added %*	Hours	Mannose recovered %†
1·44	12	6	93·4
1·20	12	6	92·4
1·20	—	6	91·8
0·62	9	6	92·4
0·60	—	6	81·5
0·60	—	2	69·0
0·56	12	6	90·0
0·37	16	6	86·0
0·30	—	2	51·9
0·30	—	6	71·7
0·30	9	6	83·9
0·15	—	2	8·0
0·15	—	6	38·0
0·075	—	4	Nil
0·075	—	6	Nil

10 % phenylhydrazine hydrochloride solution containing equivalent sodium acetate. 10 ml. sugar solution used in each case.

Conc. of mannose %	ml. phenyl-hydrazine-HCl solution	Hours	Mannose recovered %†
2·1	5	6	94·0
2·1	10	6	95·4
2·1‡	5	6	Nil
1·5	5	6	94·7
1·2	5	6	95·3
1·2	5	24	94·9
1·2	5	6§	91·7
1·2	5	3	93·2
0·6‡	5	6	Nil
0·5	5	6	88·3
0·38	5	6	86·5
0·28	5	4§	70·5
0·28	5	4	75·2
0·13	5	6	24·0
0·06	5	6	Nil

* The significance of the concentration of NaCl added will appear later. The amounts roughly correspond to volume of 5 % HCl selected for hydrolysis of mannan and final volume after concentration.

† Factor for calculation: hydrazone × 0·66 = mannose.

‡ With addition of 0·9 g. NaCl = 6 % NaCl on final concentration.

§ Solution heated to 75° immediately after addition of phenylhydrazine hydrochloride, then cooled to room temperature.

Several important points emerge. Either with free phenylhydrazine or the hydrochloride the recovery of mannose is dependent on the initial concentration of sugar in the mixture and only approaches a quantitative figure when the mannose concentration is about 1 % or more. At very low concentration no precipitate forms. The time of standing must be longer than the 2 hours recommended by Schorger [1917], but 6 hours appear to be sufficient if the mixture be frequently shaken. It has recently been claimed that, in low concentrations of mannose, it is necessary to allow 3 weeks for the precipitate to form [Nishida *et al.* 1936]. No advantage was gained by heating to 75° after addition of the phenylhydrazine, though the precipitated hydrazone appeared sooner. The effect of the presence of salt is difficult to understand. With phenylhydrazine in the proportions stated, the presence of salt increases the recovery of mannose so that the limit of mannose concentration for 90 % recovery or better is lowered to 0·5–0·6 %. Using the hydrochloride, and under conditions in which the excess of phenylhydrazine is distinctly less, the addition of salt prevents the formation of any precipitate even on standing for 24 hours.

In view of these findings, some of the figures given by Schorger [1917] for the total mannan content of wood cannot be considered reliable. If, as stated, only 10 g. of wood were used for hydrolysis, and the final precipitation was carried out in 150 ml., the recoveries of mannose must have been incomplete in many cases.

The mannan content of pulps has been determined by Klingstedt [1933], who avoided the difficulties brought about by low concentrations by hydrolysing the pulp completely with 72 % sulphuric acid, boiling after dilution and concentrating to a syrup after removal of sulphuric acid. Even so the conditions of the final precipitation do not result in recoveries of mannose so nearly quantitative as obtained in this work.

There is, however, need for further investigation of the precipitation of mannose as the hydrazone in order to obtain a more sensitive method suitable for woods. It was hoped to employ the iodimetric titration devised by Ling and Nanji [1921] for the determination of small quantities of the hydrazone, but the incompleteness of the precipitation rendered this impossible. Substituted phenylhydrazine derivatives might prove more suitable in low concentration and will be tested.

II. *The distribution of mannan in wood.*

The method adopted for the determination is as follows. 10–15 g. of finely ground wood, or 35–40 g. of wet wood cellulose, are boiled with 100 or 150 ml., 5 % HCl under reflux condenser for 3½ hours, the residue after filtration on cloth being thoroughly washed twice with about 300 ml. hot water. The combined extract and washings, after cooling, is neutralized with NaOH, made just acid with acetic acid and concentrated to about 25–30 ml. The concentrated solution is filtered into a small marked flask and the filter paper washed till the volume is made up to 50 ml. (or 75 ml. in some cases). After cooling 2·5–4 ml. phenylhydrazine and up to 5 ml. 50 % acetic acid are added. The flask is closed and allowed to stand for 6 hours at room temperature with constant shaking. The final volume is noted, the hydrazone is filtered off on a Gooch crucible, washed with about 50 ml. cold water followed by acetone, dried and weighed. Hydrazone × 0·6 = mannan or × 0·66 = mannose.

A bulky precipitate due to the salting out of phenylhydrazine acetate may form if too much acetic acid is used, and additional water may have to be added. A small amount of this precipitate has no influence on the hydrazone yield and is removed on washing with water.

In all determinations the results have been rejected if the final concentration of mannose in the solution from which the hydrazone was precipitated fell below 0·5 %, and further determinations were made employing larger quantities of wood or cellulose. The period of 3½ hours' hydrolysis has been based on Schorger's observation and the knowledge that both encrusting hemicelluloses and cellulosans are hydrolysed in that time. If small amounts of mannan remain unhydrolysed they cannot be detected by a second hydrolysis because they fall below the limits of quantitative precipitation. 5 % H_2SO_4 has been employed for hydrolysis and the acid subsequently removed with barium carbonate. This process, though very desirable, results in losses of mannose.

To study the distribution of the mannan in wood, the total mannan and the mannan associated with the cellulose were separately determined (Table II), the cellulose preparations and determinations being made by the procedure described by Norman & Jenkins [1933]. All results are the average of two closely agreeing determinations. In view of the fact that, as shown in Table I, completely quantitative recovery of mannose is not obtained under the conditions of precipitation employed, the figures must err slightly on the low side. The woods analysed may be taken as a representative group of softwoods. In all cases the major part of the mannan was found to be associated with the cellulose. The highest content occurred in Douglas fir, and in this wood, and in Redwood, virtually all the mannan was present as cellulosan. In the final column of Table II is given "mannan not in cellulose", obtained by difference. It is unlikely that this has any existence in fact as a true mannan, but is probably a constituent part of the encrusting hemicelluloses. As expected, no regularity was found in the proportions of mannan to xylan in the cellulose. The variation was very wide, from Douglas fir cellulose on the one hand, which contained nearly twice as much mannan as xylan, to African pencil cedar on the other, with more than twice as much xylan as mannan. The number of samples is insufficient to show whether

Table II. *Distribution of mannan in wood.*

	Expressed on wood %		Expressed on cellulose %		Expressed on wood %		
Wood	Total mannan*	Cellulose	Mannan in cellulose*	Xylan in cellulose	Mannan in cellulose	Xylan in cellulose	Mannan not in cellulose
Sitka spruce (*Picea sitchensis*)	6·02 (0·89)	61·87	8·43 (1·53)	5·05	5·22	3·12	0·8
Canadian spruce (*Picea* sp.)	5·75 (0·98)	63·72	4·92 (0·86)	9·19	3·13	5·86	2·6
Douglas fir (*Pseudotsuga taxifolia*)	7·06 (1·17)	57·46	10·14 (1·72)	5·54	6·82	3·18	0·2
Silver fir (*Abies* sp.)	5·96 (0·63)	52·90	7·01 (0·93)	7·34	3·70	3·88	2·3
Pitch pine (*Pinus* sp.)	5·07 (0·92)	55·88	7·01 (1·21)	8·30	3·92	4·64	1·2
Louisiana Gulf cypress (*Tarodium distichum*)	2·97 (0·55)	50·55	3·84 (0·53)	7·99	1·94	4·04	1·0
African pencil cedar (*Juniperus procera*)	2·40 (0·52)	44·29	4·05 (0·65)	11·03	1·79	4·89	0·6
Redwood (*Sequoia* sp.)	2·62 (0·72)	49·08	4·77 (1·08)	11·61	2·34	5·70	0·3

* Figures in brackets give the mean concentration of mannose in the final solutions from which the hydrazone was precipitated.

this may be a varietal difference, or influenced by age or tissue. Norman [1936, 1] has shown that the amount of xylan associated with the cellulose of rye grass increases with the age of the tissue. If there is any degree of constancy in the mannan/xylan ratio for particular species, this might prove a useful method of identification in certain cases. The cellulosan content of these softwood celluloses, as judged by the mannan and xylan contents combined (12–16 %), is notably lower than that of hardwood celluloses in which it is often over 20 %. This fact is of course represented in another way in the higher α-cellulose content of the softwood celluloses.

III. *Hydrolysis and extraction of mannan from softwood cellulose.*

The hydrolysis of wood with dilute mineral acids has been extensively studied, but few investigators have examined the behaviour of isolated celluloses. Hawley & Fleck [1927] reported that all the mannan and xylan could be easily removed from spruce cellulose by the action of dilute acid.

The purpose of the experiments described below was to study the relative behaviour of mannan and xylan to hydrolytic and extracting agents, since this has an important bearing on the form of association of the cellulosan with the true cellulose component of the wood cellulose. The wood employed was Silver fir, which seemed particularly suitable for this purpose inasmuch as the mannan and xylan contents of the cellulose are almost identical.

A large preparation of cellulose was made from this wood, and in view of the observation that drying may change the relationship between cellulose and cellulosan [Norman, 1936, 2], all treatments were carried out on the wet cellulose. The losses resulting from the different treatments were determined on small samples of cellulose under the same conditions as used for the larger amounts needed for mannan determination. The residues from these smaller quantitative experiments were later utilized for the determination of xylan by furfuraldehyde yield. In the case of the experiments on acid hydrolysis mannan was determined on the extract or the residue, whichever provided the highest concentration. It proved difficult to select such amounts as would keep the concentration above the limits necessary for maximum recovery. The results of hydrolysis with dilute acid are given in Table III.

Table III. *Hydrolysis of Silver fir cellulose with boiling dilute HCl.*

Mannan in cellulose 7·01 %. Xylan in cellulose 7·34 %.
Results expressed as % of oven-dry cellulose.

Treatment	Residue	Mannan in residue*	Xylan in residue	Hexosan removed
3 % HCl 1 hour	84·91	4·16 (0·74)	4·78	9·68
3 % HCl 3 hours	77·45	2·78 (0·44)	2·93	13·91
1 % HCl 1 hour	90·09	5·29 (0·75)	5·16	6·01
1 % HCl 3 hours	86·40	3·76 (0·42)	4·78	7·79
0·25 % HCl 1 hour	94·94	6·62 (0·83)	5·84	3·17
0·25 % HCl 3 hours	91·74	5·47 (0·67)	5·04	4·42

* Figures in brackets give the mean concentration of mannose in the final solutions from which the hydrazone was precipitated.

The removal of both mannan and xylan was far from complete even after boiling with 3 % acid for 3 hours. The mannan and xylan showed little difference in behaviour excepting perhaps to the lowest concentration, 0·25 %, towards which the xylan appeared to be more susceptible than the mannan. Attention is drawn again to the last column of the table in which the amount of material removed which is not mannan or xylan, but presumably glucosan, is recorded. In every case this is greater than the combined loss of mannan and xylan.

Interesting differences in behaviour of the xylan and mannan were shown in cellulose exposed to cold and hot alkali (Table IV). The mannan appears to be more resistant to extraction than xylan when cold alkali is employed, but distinctly less resistant to hot alkali. 1% NaOH at the boil for 20 min. removed 60% of the mannose and only 6% of the xylan whereas cold 4% NaOH extracted about 55% of the xylan as against 21% of the mannan. The significance of these differences is not apparent but is worthy of more extended study. In the preparation of mannan from cellulose the use of hot 1% NaOH for a short time would result in a purer extract than would be obtained with a higher concentration. The results are, of course, insufficient for generalization, but suggest that the stabilities of mannan and xylan are of the same order and the forms of association with the cellulose of a similar type.

Table IV. *Treatment of Silver fir cellulose with NaOH both hot and cold.*

Mannan in cellulose, 7·01%. Xylan in cellulose, 7·34%.
Results expressed as % of oven-dry cellulose.

Treatment	Residue	Mannan in residue*	Xylan in residue	Hexosan removed
4% NaOH 2 hours (room temp.)	92·07	5·50 (0·90)	3·30	2·24
1% NaOH 2 hours (room temp.)	98·02	6·71 (0·94)	6·67	1·01
4% NaOH 20 min. (boiling)	85·43	2·30 (0·52)	4·96	7·48
1% NaOH 20 min. (boiling)	89·86	2·82 (0·56)	6·90	5·51

* Figures in brackets give the mean concentration of mannose in the final solutions from which the hydrazone was precipitated.

SUMMARY.

1. The precipitation of mannose as the phenylhydrazone is not quantitative at very low concentrations. Almost complete recovery may be obtained if a certain minimum concentration is exceeded. Salt may aid precipitation if a large excess of phenylhydrazine is present.

2. Conditions suitable for the determination of mannan in woods and wood cellulose are described.

3. The major part of the mannan in softwoods is associated with the cellulose. Considerable variation is found in the proportion of mannan to xylan occurring in the cellulose.

4. Mannan may be removed from cellulose by dilute acid hydrolysis under conditions similar to those in which the xylan is extracted. At the same time there is a considerable loss of hexosan. The mannan and xylan are affected to different extents on treatment of the wood cellulose with alkali.

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CCCV. THE NUTRITION OF *STAPHYLOCOCCUS AUREUS*. NECESSITY FOR URACIL IN ANAEROBIC GROWTH.

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(*Received 31 October 1936.*)

Aerobically, *Staphylococcus aureus* has been grown [Fildes *et al.* 1936] on a mixture of nutrients of which only one component was unknown [Knight, 1935]. This medium would not however permit anaerobic growth. For growth anaerobically, it was necessary to add pyruvic acid and a second unknown substance (Factor III, Fildes *et al.* [1936]). By a process of fractionation, confirmed by synthesis, this substance is now identified as uracil.

Experimental.

The general bacteriological technique is described elsewhere [Fildes *et al.* 1936]. Any details omitted here may be found in that paper.

Conditions of test.

The object of early work was the isolation of the unknown substance from crude marmite. In consequence the bacterial test was designed, at first, to show the distribution of activity after a given chemical fractionation. The fractions obtained were tested by addition to the standard medium in serial five-fold dilutions; dilutions giving comparable growths in 20–24 hours were considered to have similar contents of active material. In this way the result of any fractionation could be estimated. For later work on the inherent activity of purines, pyrimidines and other cyclic nitrogenous compounds, however, experiments had to be of longer duration. It soon became obvious that traces of growth might occur after a delay of 3 days without addition of any active substance. The reason for this has not yet been fully determined, but it was possibly due in part to a failure in anaerobic technique.

Anaerobic technique. With *Staph. aureus* growing anaerobically, oxygen is an impurity of which the merest trace may vitiate an experiment. The organism, without uracil, is under a restriction which it may escape if air is present. There is, in consequence, an extreme sensitiveness to traces of oxygen. To ensure uniformity of atmosphere in any one experiment, use was made of the McIntosh and Fildes anaerobic jar. For each filling, however, it was thrice exhausted on a Geryk pump and refilled with hydrogen + 5 % CO_2 [Gladstone *et al.* 1935] before warming the platinized asbestos capsule. Occasionally, separate platinized asbestos mats were also added. If an experiment was to continue after the first reading, opacities were estimated and the tubes returned to anaerobic conditions as quickly as possible; any subsequent growth was always regarded with some suspicion. For the more exacting work, an experiment in its early stages was observed through the jar without opening it.

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[1] Leverhulme Trust Fellow.

Nevertheless, these procedures could not be wholly relied on to eliminate traces of visible growth from the control. Since the atmosphere of the jar seemed free from oxygen according to indicators, it was concluded that oxygen might be dissolved in (or combined with) the medium itself. A small amount of O/R dye was therefore added to the medium; since the latter already contained a ferrous salt, the reduction of dye which was apparent in a few hours was considered to indicate the removal of oxygen. This procedure resulted in controls which were more nearly satisfactory. Nevertheless, the slight, delayed growths without uracil did not seem wholly attributable to the presence of oxygen. This matter will be referred to again.

Medium. The medium is described elsewhere [Fildes *et al.* 1936]. Its organic components were: amino-acids, glucose, pyruvic acid, an organic sulphur compound and the factor described by Knight [1935]. The organic sulphur compound was specified as dithiodiglycollic acid but in most of this work thiolacetic acid was used. As stock solution, a $M/10$ solution in M HCl was autoclaved and was stable for weeks. 0·2 ml. was added per tube together with 0·25 ml. M NaOH, making the concentration of —SH in the experiment $M/500$. Comparative tests have not shown any difference in the effect of uracil whether in the presence of —SH or —SS—. $M/250{,}000$ indigotetrasulphonate was used as indicator regulating the oxygen effect, on account of its lack of toxicity in immersion experiments [Chambers *et al.* 1931].

Preparation of test samples. Fractions for test were freed from any toxic materials used in fractionation, adjusted roughly to *p*H 7·6, brought to a dilution equivalent to a 1 % solution of the original crude marmite, autoclaved and tested in fivefold serial dilutions.

Distribution of accessory substance.

Of raw materials tested, crude marmite was active at highest dilution in permitting growth. "Bacto peptone" and aqueous extracts of liver and malt showed moderate activity, whilst minced meat and pea extracts, and caseinogen, gelatin, egg albumin and edestin hydrolysates showed little or none. It is now seen that this distribution coincides with the probable distribution of nucleic acid and similar substances in the crude extracts.

Chemical behaviour of accessory substance from crude marmite.

As present in crude marmite, the substance was stable to autoclaving and to $3N$ H_2SO_4 (8 vol. %) either at 130° for 3 hours or during 18 hours' boiling. Its activity, in fact, was enhanced by acid hydrolysis. It was incompletely destroyed by 18 hours' boiling with $3N$ NaOH or $Ba(OH)_2$. It was stable to heating at 200° for 5 min. but was partially destroyed at 250° in 15 min. It was not distillable in a high vacuum at the latter temperature.

It was not extractable by ether from aqueous solution at any *p*H and only with difficulty by *n*-propyl and *n*-butyl alcohols. The latter extraction proceeded better (on hydrolysed marmite) at *p*H 1 or 6 than at *p*H 10, and six extractions under the former conditions removed about half the activity. Continuous extraction by the Dakin method proceeded rather slowly. An attempt to utilize the apparent weakly acidic properties of the substance in order to transfer it from aqueous marmite to N Na_2CO_3 solution by continuous rocking under a layer of butyl alcohol was only moderately successful. It was however possible, by shaking with equal volumes of aniline and alcohol, to extract 40 % of the activity in one shaking into the aniline-alcohol layer. From dry "Bacto peptone", the activity could be partially removed by continuous extraction with absolute alcohol for 12 hours. Progressive addition of alcohol to strong solutions of hydrolysed or unhydrolysed marmite produced merely a progressive precipitation of activity.

The active material was not significantly precipitated by $Ba(OH)_2$, lead acetate (at *p*H 6) or basic lead acetate (at *p*H 7·4, Britton & Meek [1932]). It was partially precipitated by $Ba(OH)_2$ followed by 75 % alcohol. It was not notably adsorbed upon fuller's earth, alumina, kieselguhr or frankonite at *p*H 6, or upon norite at any *p*H. It was partially precipitated by phosphotungstic acid in 5 % H_2SO_4. It was not precipitated by mercuric acetate or chloride in aqueous or 50 % alcoholic solution, or by the sulphate in 5 % H_2SO_4. It was, however, precipitated by acid $HgSO_4$ in 66 % alcohol, by $HgCl_2$ in absolute alcohol and by Neuberg's mercuric acetate-sodium carbonate reagent for amino-acids.

At this stage therefore the possibility of distinguishing between the active material and those amino-acids which comprise about 40 % of the crude marmite seemed quite remote. Indeed, it seemed feasible, in view of the acknowledged importance of amino-acids in the nutrition of bacteria [Knight, 1936] that we were dealing with some uncommon amino-acid not present in proteins. In opposition to this view, the activity was not destroyed by nitrous acid; but the product of such treatment might easily have had similar biological activity.

Preparation of active concentrates from crude marmite.

There were then two methods which might give a practical separation of the active material from the bulk of the amino-acids: precipitation by acid $HgSO_4$ in 66 % alcohol and serial extraction by aniline-alcohol. Owing to the difficulty of maintaining the right proportions in an extraction by a ternary liquid mixture, the former method was selected.

A strong solution (dry weight 50 %) of marmite was precipitated with 3 vol. absolute alcohol and left overnight at 0°. The clear supernatant liquor, though of considerably diminished activity, was in a more workable condition. It was treated with 25 % $HgSO_4$ in 10 % (by vol.) H_2SO_4, until the curdy precipitate gave place to a somewhat granular one, and filtered. The precipitate was suspended in water, freed from Hg^{++} and $SO_4^{=}$, and concentrated. It was then brought to *p*H 4 and fractionated by the $AgNO_3$-$Ba(OH)_2$ method for imino-compounds. The active material was in the pyrimidine-glyoxaline fraction.

The medium already contained histidine, and it was soon demonstrated that thiolhistidine also had no effect. The naturally occurring pyrimidines and purines were therefore submitted to test.

Preparation from yeast nucleic acid.

Commercial preparations of yeast nucleic acid and of cytidylic acid both contained much active material, the activity being enhanced on acid hydrolysis in the usual way. By fractionation of the acid-hydrolysed nucleic acid with $AgNO_3$-$Ba(OH)_2$, the active material was again obtained in the pyrimidine fraction. Treatment of the latter with picric acid left the bulk of the activity in the uracil fraction. On the other hand, both picrates and flavianates from the pyrimidine fraction (which presumably contained traces of guanine, adenine and hydrolytic products therefrom, in addition to cytosine), showed quite appreciable activities. Likewise, a commercial guanine preparation showed some activity, though an adenine preparation had none. It was clear that a final decision on the relative importance of various cyclic compounds of this type could only be reached by using synthetic materials.

Uracil as the active substance.

In Table I are shown the effects of selected pyrimidines, purines and other cyclic nitrogenous compounds, mostly synthetic materials, on the strictly anaerobic growth of *Staph. aureus*. Clearly, only uracil is fully active in permitting anaerobic growth. If the other compounds can be utilized (and it is unwise, even with controls, to draw conclusions from delayed growths which have been exposed to air) the organism is considerably inconvenienced in using them. It is possible that the evidence of summation in the utilization of the four bases has significance, but even there, the organism is severely limited by its lack of uracil.

Table I. *Effect of uracil and related substances on the anaerobic growth of* Staph. aureus *on the basal medium.*

Compound	Constitution (enolic tautomeride)	Growth after 24 hr.		Growth after 44 hr.		Growth after 68 hr.
Control	—	0†		0	↑	0
Control	—	0		0		0
Control	—	0		0		0
Uracil,* *M*/20,000	2:6-Dihydroxypyrimidine	+ +		+ + +	↓	+ + +
M/100,000	,,	tr.	↑	+ +		+ +
M/500,000	,,	?		+ +		+ +
M/2,500,000	,,	0		+		+
Thymine,* *M*/20,000	5-Methyluracil	0		0		tr.
M/100,000	,,	0		0		?
4-Methyluracil,* *M*/20,000	4-Methyluracil	0		0		+
1:3-Dimethyluracil,* *M*/20,000	1:3-Dimethyluracil	0		0		tr.
1:3:4-Trimethyluracil,* *M*/20,000	1:3:4-Trimethyluracil	0		0		tr.
Thiothymine,* *M*/20,000	2-Thio-5-methyluracil	0		0		tr.
Barbituric acid, *M*/20,000	4-Hydroxyuracil	0		0		tr.
Barbitone, *M*/20,000	(5:5-Diethylbarbituric acid)	0		0		–
Xanthine, *M*/20,000	2:6-Dihydroxypurine	0		0		+
Theobromine, *M*/20,000	3:7-Dimethylxanthine	0		0		tr.
Uric acid, *M*/20,000	2:6:8-Trihydroxypurine	0		0		tr.
Guanidine HCl, *M*/20,000		0		0		+
Urea, *M*/20,000		0		0		tr.
Control		0	↓	0		?
Pyrimidine and purine bases.						
Cytosine,* *M*/20,000	2-Hydroxy-6-aminopyrimidine	0		0	↑	tr.
*iso*Cytosine,* *M*/20,000	6-Hydroxy-2-aminopyrimidine	0		0		0
Guanine,* *M*/20,000	6-Hydroxy-2-aminopurine	0		0		tr.
Adenine, *M*/20,000	6-Aminopurine	0		0		0
4 Bases as above, *M*/20,000 (each)		0		+		+ +
4 Bases with uracil, *M*/20,000 (each)		+ +		+ + +	↓	+ + +
Vitamin B_1, *M*/17,000	Derivative of 6-aminopyrimidine	0		0		–
Other cyclic nitrogenous compounds.						
Thiolhistidine,* *M*/2000		0		–		–
Hydantoin,* *M*/20,000		0		0		?
Allantoin, *M*/20,000		0		0		?
Creatinine, *M*/20,000		0		0		?

* Substance of known synthetic origin.

† + + + = maximum growth, other plus signs roughly proportional. tr. = just visible opacity. ? = dubious opacity in comparison with 0 (none). – = not observed.

↕ = jar opened. Tubes returned to anaerobic conditions before O/R indicator was visibly coloured.

Uracil was synthesized by the method of Davidson & Baudisch [1926], simplified by dissolving the malic acid and urea (1 part of each) in 2 parts warm conc. H_2SO_4 before adding 8 parts of fuming H_2SO_4 (20% SO_3). Yields were comparable (53%). (Found: N (micro-Kjeldahl with selenate catalyst and added glucose), 24·9%. $C_4H_4O_2N_2$ requires 25·0%.)

Cytosine and isocytosine were synthesized from uracil by the methods of Hilbert *et al.* [1935]. (Cytosine. Found: N, 32·4%. $C_4H_5ON_3.H_2O$ requires 32·6%. *Iso*cytosine (known to be contaminated with cytosine). Found: N, 36·4%. $C_4H_5ON_3$ requires 37·8%.)

Guanine was synthesized from guanidine thiocyanate and ethyl cyanoacetate by the method of Traube & Dudley [1913]. (Guanine. Found: N, 46·0%. $C_5H_5ON_5$ requires 46·4%.)

Thymine was synthesized from thiourea and ethyl α-formylpropionate by the method of Wheeler & MacFarland [1910]. To minimize the possibility of final contamination by uracil, the dry ethyl propionate in the initial stage was fractionated and the first half of the distillate discarded. The 2-thiothymine formed in the intermediate stage was recrystallized from water. (Found: N, 21·9%. $C_5H_6O_2N_2$ requires 22·2%.)

Hydantoin, 2-thiolhistidine, and the methylated uracils were synthetic materials kindly supplied by Prof. C. R. Harington and Dr H. R. Ing.

Vitamin B_1 was a crystalline preparation for which we are indebted to Prof. R. A. Peters.

Synthesis of active substance by Staph. aureus *growing aerobically.*

60 ml. of a 3-day aerobic culture of *Staph. aureus* on the aerobic medium without uracil [Fildes *et al.*, 1936] were centrifuged, and the supernatant liquor decanted for test for active substance after sterilization by filtration. The bacterial matter was mildly hydrolysed by boiling for 90 min. with *M* HCl, neutralized, diluted to 30 ml. and also tested for active substance (Table II).

Table II. *Synthesis of active substance by* Staph. aureus *growing aerobically.*

	Growth in 3 days
Control	0
Control	0
Filtrate (diluted 1/10)	+
Filtrate (diluted 1/50)	0
Hydrolysed bacteria (equivalent dilution 1/5)	+ +
Hydrolysed bacteria (equivalent dilution 1/25)	+
Hydrolysed bacteria (equivalent dilution 1/125)	0
Control (*M*/20,000 uracil)	+ + +

Clearly, a substance having the growth-promoting properties of uracil is synthesized by *Staph. aureus* aerobically. Chemical identification of the uracil, however, must be deferred until the amino-acid nutrition of the organism has been adequately studied and simplified.

The synthesis of active substance thus demonstrated under aerobic conditions may have some bearing upon the delayed growths without uracil under anaerobic conditions which have been referred to above. It is obvious in Table I that growth of *Staph. aureus* without uracil was in no way comparable with its growth with uracil. If this need for added uracil is due to failure to synthesize it, the failure need not, of course, be absolute. In this event, traces of growth without uracil might reasonably be expected.

Synthesis of active substance is also accomplished by *Bact. typhosum* growing aerobically on ammonium lactate medium. Indeed, the general phenomenon seems comparable with that, for instance, of the metabolism of tryptophan. This amino-acid is synthesized by those bacteria which by nature or training can grow without a supply of this important nutrient [Fildes & Knight, 1933].

DISCUSSION.

It has been pointed out elsewhere [Fildes & Richardson, 1935] that the criterion of indispensability adopted for work on bacterial nutrition differs in kind from that adopted in animal nutrition. In studying the growth of our small inoculum (dry weight about 4×10^{-6} mg.) we are dealing with a successful propagation through many generations and an increase of weight of about a millionfold. Any substance which is a limiting factor in this propagation is therefore probably of deep significance to cellular nutrition in general.

Uracil is interesting, therefore, not alone in relation to the growth of *Staph. aureus* growing anaerobically. It is synthesized in the aerobic growth of both *Staph. aureus* and *Bact. typhosum* (the only other organism tested), and may be presumed to have an important function in bacterial metabolism in general. It would be premature to speculate what this may be. One may note, however, that although adenylpyrophosphate and cozymase (two adenine nucleotides) are both coenzymes active in various phases of hexose metabolism, no function has yet been assigned to other nucleotides. It is possible that a nucleotide derived from uracil exerts an effect in the metabolism of *Staph. aureus* which no derivative of adenine or like substances can exert.

The discovery that this anaerobic growth substance is actually uracil, moreover, suggests that uracil has a wide distribution in nature. This view contrasts with the older supposition that uracil is not a component of animal tissues, though characteristic of plants. It also confirms the argument of Levene [1926] that uracil is indeed a primary constituent of tissues and not a product of the deamination of cytosine by the process of isolation [Jones & Perkins, 1925]. Uracil (or its derivatives) will probably be found widely distributed in bacteria. It is present in beer yeasts and is a primary component of at least the liver of certain mammals.

Of course this argument rests on the presumed specificity of the bacterial test for uracil. As far as we have been able to show, this test is at least as specific as the more common colour reactions. It is, moreover, highly sensitive (Table 1) and, we consider, is a valuable micro-test for uracil where none has been previously available.

SUMMARY.

Anaerobic growth of *Staph. aureus* upon a mixed amino-acid medium containing pyruvic acid and other necessary nutrients [Fildes *et al.* 1936] was only possible, under the conditions of test, upon addition of uracil (2:6-dihydroxypyrimidine). Of twenty-one related compounds which were tested, none had a comparable effect in permitting growth. The significance of this to the distribution and function of uracil in nature is briefly discussed.

I am greatly indebted to Dr P. Fildes for his active help and advice throughout this work, and to the other members of this department for assistance at all stages. I wish to thank the Marmite Food Extract Co., Ltd., for generous supplies of crude marmite and Dr O. Rosenheim for some specimens of natural bases.

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Note added 30 November 1936. Preliminary tests with 5 further strains of *Staph. aureus* confirm that the basal medium was adequate for aerobic, but not for anaerobic, growth. Anaerobically, addition of uracil permitted growth of 3 strains, whilst hydrolysed nucleic acid was required for the remaining 2 strains. Of the latter, the only one tested depended upon guanine and uracil for good growth. The species in general seems, therefore, to need various constituents of nucleic acid according as its synthetic powers are limited by the conditions of anaerobic growth.

CCCVI. A MODIFIED COLORIMETRIC ESTIMATION OF CARBONIC ANHYDRASE.

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(*Received 19 August 1936.*)

INTRODUCTION.

The work described here is the result of an attempt to devise for class purposes a method of estimating carbonic anhydrase which does not involve the use of special apparatus as in the boat method of Meldrum & Roughton [1933]. It is a modification of the colorimetric method of Brinkman [1933], who mixed solutions of CO_2 and $NaHCO_3$ plus phenol red and measured the time required for the colour change.

In our method the $NaHCO_3$ is replaced by Na_2CO_3 (plus a little $NaHCO_3$). The reaction begins at *p*H about 10·5, in the middle of the $NaHCO_3$-Na_2CO_3 buffer range, and the end-point is taken at *p*H 7·0, using bromothymol blue as an indicator. The reaction therefore begins slowly in the well-buffered part of the titration curve and ends on the steep part of the curve, thus producing a rapid and very sharp end-point. The net reaction is approximately:

$$CO_2 + CO_3'' + H_2O \rightarrow 2HCO_3'.$$

The chief merit of the changes introduced is probably the dramatic suddenness of the end-point, which not only permits great accuracy of timing but also is an asset for class purposes. The method gives good results for moderately purified enzyme preparations, but for other cases (notably for enzyme estimations in tissue extracts) it has certain drawbacks which make it compare unfavourably with the boat method. These are:

(*a*) The detailed mechanism of the reaction alters during its course, owing to the *p*H change.

(*b*) The choice of ionic strength of the solutions used is restricted by several practical considerations.

(*c*) The reaction time can be shown theoretically to be rather sensitive to extraneous buffering in the *p*H region 7–9.

(*d*) Haemoglobin has a curious effect on the calibration curve which is described below.

From the research point of view therefore the applicability of the method is probably limited to routine estimations under fairly constant conditions, or to problems involving purified enzyme preparations. For these however the rapidity (15 estimations per hour) and ease of manipulation will be found advantageous.

EXPERIMENTAL.

Solutions. (*a*) Na_2CO_3. 0·3*M* solution, containing 0·206*M* $NaHCO_3$. The latter was added to keep the reaction from becoming too alkaline since carbonic anhydrase is only stable from *p*H 4 to 11. This solution was prepared from a solution saturated with Na_2CO_3 and $NaHCO_3$ at room temperature by diluting to 27·3 parts in 100. The carbonate was estimated by titration with HCl to *p*H 8·3, running in the HCl under the surface with rapid stirring, to avoid loss of

CO_2, and waiting about 30 sec. after each addition. The bicarbonate was estimated by titrating to *p*H 4·0 and subtracting twice the carbonate. The indicators used were cresol red and bromophenol blue respectively.

(*b*) CO_2. A 0·00263*M* solution of $NaHCO_3$ was saturated with CO_2 by bubbling from a cylinder. The $NaHCO_3$ was added to keep the initial reaction from being too acid and destroying the enzyme.

(*c*) Enzyme. A crude preparation, made from pig corpuscles by the $CHCl_3$ method, was used, diluted 1/50. (This preparation was kindly supplied by Mr V. H. Booth.)

(*d*) Indicator. Bromothymol blue.

APPARATUS.

Fig. 1 shows the final set-up. Most of the accessories are unnecessary for rough work.

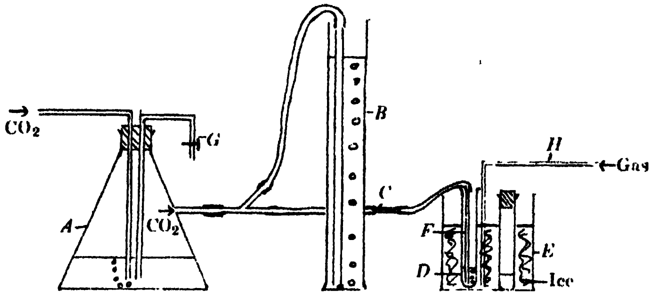

Fig. 1. Diagram of apparatus. *A*, reservoir of CO_2 solution; Büchner flask. *B*, pressure regulator (bubbling from bottom of wide tube). *C*, fine capillary, about 5·5 cm. long. *D*, reaction vessel; pyrex boiling-tube 2·5 cm. diameter. *E*, vessel for colour standard. *F*, CO_2 bubbling tube. *G*, clip for obtaining CO_2 solution from reservoir. *H*, gas bubbler to stir the ice and water surrounding the reaction vessel (air or any gas will do). The flask *A*, tubes *D* and *E*, the reagents and clean reaction tubes are all kept in ice and water.

PROCEDURE.

x ml. enzyme and $11-x$ ml. CO_2 solution are measured with pipettes into the cooled reaction tube. (The CO_2 solution is run from *G* into a test-tube and then measured accurately with a pipette.) Ten drops of indicator and one drop of octyl alcohol (to prevent frothing) are added and CO_2 is bubbled through. After 2 min. 1 ml. of Na_2CO_3 is blown in from a graduated pipette and the time is measured for the colour to change from blue to a yellow-green. CO_2 is bubbled the whole time. The end-point is judged by matching the colour with the standard tube which contains phosphate buffer at *p*H 7·0 and the same indicator. With reaction times below 30 sec. the end-point is so sharp that exact matching is out of the question.

PRECAUTIONS.

The 2 min. of preliminary bubbling are to ensure saturation with CO_2 and to allow temperature equilibrium to be reached. If the surrounding ice and water are kept well stirred this is quite long enough.

It was found that the reaction time was affected by the rate of bubbling of CO_2 during the reaction. This was due to

(*a*) the effect on the rate of the initial mixing,

(*b*) the fact that CO_2 continues to dissolve during the reaction to replace that which is used up.

The two factors concerned were the rate of flow of CO_2 and the nature of the bubbling tube *F*.

The rate of flow was standardized by introducing the pressure regulator *B* and the capillary *C*. We finally decided to define the conditions as follows:

Bubbler: length 19 cm.: cross-section 4·5–6·5 sq. mm.

Rate of flow: 70–90 ml. in 15 sec.

This gives a reaction time nearly as short as that obtained with much larger rates of flow. The rate of flow is adjusted by (*a*) the length and bore of the capillary *C*, and (*b*) the head of water in the pressure regulator *B*.

It was found that, within these limits, variations in the above conditions caused less than 5 % systematic error, since the total error, both random and systematic, was never more than 5 %.

Definition of unit. If the reciprocal of the reaction time is plotted against the amount of enzyme the result is a straight line within the useful range (time: 11–40 sec.). Thus the number of enzyme units in a solution giving a reaction time t is $K\ (t_0/t-1)$, where t_0 is the reaction time of the blank (54 sec.) and K is a constant equal to 17·7. The value of K was found empirically by comparing our measurements with data kindly obtained for us by Mr V. H. Booth on the same preparation by means of the boat method.

If the purified enzyme is replaced by haemoglobin or defibrinated blood the calibration curve is shifted along the concentration axis, with a small non-linear region near the origin. Corrections for this can be made, but it is better not to attempt absolute measurements in the presence of haemoglobin.

SUMMARY.

A modified colorimetric estimation of carbonic anhydrase is described, capable of 15 estimations per hour, with an accuracy of about 5 % over a two-fold range of enzyme concentration.

The method requires no special apparatus and has, so far, been tested on moderately purified enzyme preparations and on haemoglobin solutions. In other cases further controls would be necessary.

We are grateful to Prof. R. A. Peters for his interest in this work.

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CCCVII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

LII. ISOLATION, PROPERTIES AND CONSTITUTION OF TERRESTRIC ACID (ETHYLCAROLIC ACID), A METABOLIC PRODUCT OF *PENICILLIUM TERRESTRE* JENSEN.

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(*Received 23 October 1936.*)

IN an attempt to identify certain strains of *Penicillium* isolated from New Zealand cheddar cheese and received under the label of *P. puberulum* Bainier, Birkinshaw & Raistrick [1936] were led to examine biochemically certain strains in the *P. terrestre* Jensen series. These moulds, with the exception of one strain, when grown on a Raulin-Thom glucose medium, produced in fair quantity a hitherto undescribed metabolic product for which the name *terrestric acid* is proposed.

Terrestric acid, $C_{11}H_{14}O_4$, is a colourless, crystalline acid showing many resemblances in properties to the substituted tetronic acids obtained by Clutterbuck *et al.* [1934] from *P. Charlesii* G. Smith, and in particular to carolic acid. This similarity is evident in the following résumé of the properties of terrestric acid.

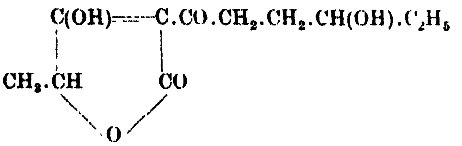
1. It gives an orange colour with aqueous ferric chloride, indistinguishable from that given by carolic acid, and with sodium nitrite it yields on standing a violet colour, a test which is indicative of the tetronic acid group. Like carolic acid it is extremely soluble in $CHCl_3$.

2. It is dextrorotatory and the molecule shows no active hydrogen in anisole but yields one atom in pyridine, although titrating sharply in water as a monobasic acid.

3. On bromination in 50% aqueous acetic acid it gives *d*-bromomethyltetronic acid identical with that obtained from carolic acid under similar conditions.

4. On acid hydrolysis it yields carbon dioxide and acetoin, but the other product is not butyrolactone as obtained from carolic acid but a *l*-hexanolactone. On oxidation with nitric acid this lactone yields succinic acid and on heating with hydriodic acid it is racemized and is then identical with *n*-hexanolactone obtained by synthesis. Part of the lactone is reduced by the hydriodic acid yielding *n*-hexanoic acid. It is clear therefore that the lactone obtained on hydrolysis is the *l*-isomeride of *n*-hexanolactone, which so far as we are aware has not hitherto been obtained in optically active form.

It thus appears that terrestric acid hydrate must have the constitution of α-(*l*-γ-hydroxy-*n*-hexanoyl)-*l*-γ-methyltetronic acid (I), whilst the anhydrous form by analogy with carolic acid [Clutterbuck *et al.* 1935] will be represented by structure II. These forms differ from the corresponding forms of carolic acid (III and IV) only in the replacement of the γ-hydroxybutyryl residue by the *l*-γ-hydroxy-*n*-hexanoyl residue. Terrestric acid may therefore be regarded as ethylcarolic acid, the ethyl group replacing a hydrogen atom of carolic acid in such a manner that another centre of asymmetry is created in the molecule.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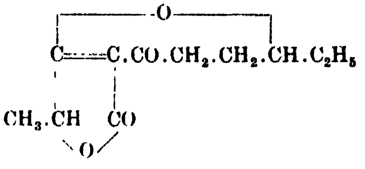
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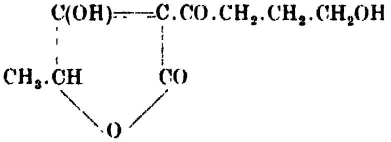
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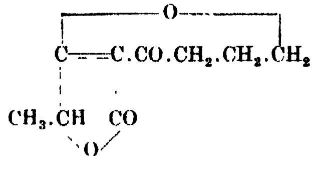
IV

EXPERIMENTAL.

Cultures.

The following strains in the *P. terrestre* Jensen series were examined:

(*a*) L.S.H.T.M. Cat. No. Ad 4. Received via Cambridge labelled *P. glaucum* (cake) and distributed originally by the British National Collection of Type Cultures under the name of *P. glaucum* Link, Cat. No. 763. Isolated from mouldy cake in 1920.

(*b*) L.S.H.T.M. Cat. No. Ad 8. Isolated by J. H. V. Charles as a bench contamination at Ardeer in 1922.

(*c*) L.S.H.T.M. Cat. No. Ad 9. Isolated by J. H. V. Charles from cheese at Ardeer in 1922.

(*d*) L.S.H.T.M. Cat. No. Ad 18. Isolated by J. H. V. Charles from milk at Ardeer in 1923–4.

All these cultures were identified by Dr Charles Thom [Birkinshaw *et al.* 1931] as strains in the *P. terrestre* Jensen series.

Cultural conditions.

The culture medium used throughout was a Raulin-Thom medium of the following composition: glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate, 4 g.; $(NH_4)_2HPO_4$, 0·6 g.: K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4$, $7H_2O$, 0·07 g.: $FeSO_4$, $7H_2O$, 0·07 g.; distilled water to 1500 ml. This was distributed in 350 ml. amounts in a number of 1-litre conical flasks, sterilized, sown with a spore suspension, prepared from Czapek-Dox glucose agar slopes, of the organism studied and incubated at a chosen temperature until the glucose content, as determined by polarimeter, had fallen below 1 %. In a preliminary experiment the yields of crude chloroform extract obtained from batches of 20 flasks of each strain when incubated at a mean temperature of 14° were Ad 4 (15 days, 0·50 % of residual glucose) 5·67 g.; Ad 8 (16 days, 0·68 % of residual glucose) 7·81 g.; Ad 9 (22 days, 0·89 % of residual glucose) 4·67 g.; Ad 18 (21 days, 0·69 % of residual glucose) 0·37 g. The chloroform extracts from strains Ad 4, Ad 8 and Ad 9 all set to a crystalline mass on standing and were all shown to contain terrestric acid, but that from strain Ad 18 did not crystallize and no terrestric acid could be detected. Strain Ad 8, which gave the best yield in this experiment, was selected for use in the preparation of terrestric acid.

Preparation of terrestric acid.

Batches of 100 flasks of Raulin-Thom medium were sown with *P. terrestre* Jensen, strain Ad 8. The flasks were incubated at room temperature either in the laboratory or in underground vaults, where a lower temperature was obtainable. The best and most consistent yields were obtained at a mean temperature of about 20°, when the period of incubation was 15–16 days, the glucose content as determined by polarimeter having fallen in that time to 0·2–0·4 %. The yield of crude crystalline material (total chloroform extract) under these conditions was about 40 g. per batch of 100 flasks. A trial batch incubated at 24° gave a somewhat lower yield and the material did not appear to crystallize with the same facility. The experimental details of a number of preparations are given in Table 1, in which all yields are calculated on a 100-flask basis.

Table 1.

Incubation period days	Temperature (° C.)		Residual glucose by polarimeter	Final *p*H of metabolism solution	Titratable acidity ml. $N/10$ NaOH per 10 ml.	Yield of crude cryst. products g.
	Range	Average				
12	24	24	0·39	3·6	1·20	37·7
15	17–21	20	0·36	3·4	1·50	41·4
16	17–21	20	0·30	3·3	1·32	39·8
16	17-21	20	0·19	3·5	1·23	39·3
24	11–18	14	0·48	2·8	1·70	34·1
21	11–18	14	0·60	2·7	2·83	28·8
19	11–18	14	0·41	2·8	2·12	35·7
19	10–14	12	0·56	2·8	2·05	25·5
35	6 12	9	0·19	3·6	0·65	39·7
32	6–10	8	0·81	3·1	2·50	18·5

When incubation was finished the metabolism solution, which gave a reddish brown colour with $FeCl_3$, was filtered from the mycelium, acidified with 10 ml. of conc. HCl per litre and extracted twice with about one-third of its volume of chloroform. The chloroform from the second extraction of each batch was used for the first extraction of a further batch. The procedure after acidification was exactly the same as that employed in the extraction of palitantin [Birkinshaw & Raistrick, 1936]. The chloroform was removed under reduced pressure and the extracts from the whole batch of 100 flasks were combined. The residual chloroform was allowed to evaporate spontaneously and in 2 or 3 days the syrup remaining set to a mass of crystals. It was usually necessary to break up the cake once or twice in order to get rid of the last traces of chloroform.

For purification the product was first crystallized from ether and then from light petroleum. Thus in a typical experiment the crude product (16 g.) was dissolved in ether (600 ml.) in two lots and allowed to crystallize. It deposited large prisms (9·22 g.) somewhat yellow in colour, M.P. 87–89°. A further crop of 2·42 g. of the same M.P. was obtained on evaporation of the ether to 50 ml. These crops were combined and boiled with light petroleum (1300 ml. B.P. 60–80°). The undissolved portion was again boiled with light petroleum (1600 ml.). This effected complete solution except for a very small dark-coloured residue. On chilling, 10·12 g. of colourless flattened needles were obtained, M.P. 89°, which represented pure terrestric acid. Since further small quantities were obtained from the mother-liquor, it is clear that most of the original chloroform extract of the metabolism solution consists of terrestric acid.

Properties of terrestric acid.

Terrestric acid is a colourless, crystalline solid of m.p. 89°, and empirical formula $C_{11}H_{14}O_4$. (Found (Schoeller): C, 62·94, 62·86: H, 6·79, 6·63%. Mol. wt. cryoscopic in dioxan (Dr A. E. Oxford) 226. $C_{11}H_{14}O_4$ requires C, 62·84; H, 6·72%; mol. wt. 210.) In aqueous solution it titrates sharply as a monobasic acid. Thus 0·0532 g. required 2·52 ml. of 0·1 *N* NaOH for neutralization to phenolphthalein, giving an equivalent of 211. Calc. for $C_{11}H_{14}O_4$ (monobasic), 210. In aqueous solution it has $[\alpha]^{20^\circ}_{5461}+61{\cdot}1^\circ$ ($c=0{\cdot}53$). It is somewhat soluble in cold water and readily soluble in most organic solvents with the exception of light petroleum, which dissolves only a trace in the cold but more on heating. Like carolic acid it is extremely soluble in chloroform even in the cold, which explains the ease with which it is extracted from dilute aqueous solution by this solvent.

In aqueous solution terrestric acid gives an orange colour with ferric chloride, indistinguishable from that given by carolic acid. It gives no immediate colour with aqueous sodium nitrite but yields a lilac colour on standing which is not so intense as that given by carolic acid under these conditions. It does not reduce Fehling's solution even on heating, gives no precipitate with neutral lead acetate, but gives in aqueous solution a precipitate with mercuric chloride.

In a Zerewitinoff determination (Roth) terrestric acid afforded no active hydrogen in anisole at 18° or 95° but gave 1 atom in pyridine at 18°.

Acid hydrolysis of terrestric acid.

Terrestric acid (0·5531 g.) was heated with 2*N* H_2SO_4 (20 ml.) under reflux in a stream of nitrogen. The bath was maintained at 135–140°. The issuing gas was passed through a bubbler containing 2:4-dinitrophenylhydrazine solution, then through standard baryta. Carbon dioxide was evolved which after 3 hours' heating was equivalent to 5·00 ml. of *N* HCl. After a further 2 hours only the equivalent of 0·17 ml. was obtained, giving a total of 5·17 ml. The calculated amount for 1 mol. of CO_2 is 5·27 ml. Hence 1 mol. of terrestric acid yields 1 mol. of carbon dioxide on acid hydrolysis. There was only a trace of precipitate in the 2:4-dinitrophenylhydrazine reagent.

The hydrolysate was cooled and titrated. It used 0·20 ml. of *N* alkali in excess of the equivalent of the H_2SO_4 used for hydrolysis. The solution was then heated and the titration continued. Further alkali (2·25 ml.) was slowly used, the titration proceeding as for a lactone. The total organic acid present was therefore equivalent to 2·45 ml., whereas the calculated amount for 1 equiv. per mol. of terrestric acid is 2·63 ml. This indicates that 1 mol. of lactone is formed per mol. of terrestric acid.

The neutralized hydrolysate was distilled *in vacuo* 7 or 8 times to dryness, water being added after each distillation to make up the loss. The distillate was made up to 500 ml. It reduced Fehling's solution in the cold and gave a precipitate with 2:4-dinitrophenylhydrazine. By analogy with carolic acid it was considered that the reducing substance present was probably an acyloin, and a Wood-Ost determination was carried out. Calculated as acetoin the total amount present in the 500 ml. was 154 mg., the theoretical amount for 1 mol. obtainable from 1 mol. of terrestric acid being 232 mg. The yield was thus 67%. Since some precipitate was present in the 2:4-dinitrophenylhydrazine bubbler employed during hydrolysis and the Wood-Ost determination was performed at great dilution, which would tend to render it inaccurate, the result was considered to indicate that 1 mol. of terrestric acid gives rise to 1 mol. of acyloin.

Identification of the acyloin. A portion of the distillate containing the acyloin was treated with ferric chloride and distilled. The distillate was treated with 2:4-dinitrophenylhydrazine. It gave overnight a copious orange precipitate which was collected and recrystallized from nitrobenzene-toluene. It gave Neuberg's reaction for a bis-dinitrophenylhydrazone and had M.P. 321°. (Found (Weiler): C, 43·19, 43·06; H, 3·11, 3·22; N, 24·88, 25·30%. $C_{16}H_{14}O_8N_8$, the bis-dinitrophenylhydrazone of diacetyl requires C, 43·04; H, 3·16; N, 25·11%.) The product showed no depression in M.P. when mixed with authentic diacetyl-bis-dinitrophenylhydrazone. Hence the acyloin formed on hydrolysis of terrestric acid is acetoin (acetylmethylcarbinol).

The residue from vacuum distillation of the acyloin was dissolved in water, acidified with H_2SO_4 and heated to 100°. After cooling it was continuously extracted with ether to remove the lactone. The ether was removed and the extracted lactone was treated with phenylhydrazine (0·3 ml.) and alcohol (1 ml.) and heated for 2 hours under reflux on the water-bath. On cooling a small amount of crystals separated. These were collected and washed with ether. M.P. 246° (decomp.). This was probably the bis-phenylhydrazone of diacetyl, which has M.P. 245° (lit.), but the amount was too small for analysis. After standing for 2 or 3 days no further crystals were observed, but on addition of ether a voluminous crop of crystals was produced, M.P. 92–93°. When recrystallized from ether the M.P. was 92°. (Found (Schoeller): C, 64·51, 64·63; H, 8·18, 8·18; N, 12·86, 12·70%. $C_{12}H_{18}O_2N_2$ requires C, 64·82; H, 8·17; N, 12·61%.) The product is thus the phenylhydrazide of a (probably γ-) hydroxyhexanoic acid.

Characterization of the lactone. In order to obtain a pure sample of the lactone for characterization, a further quantity (15 g.) of terrestric acid was hydrolysed with 2*N* H_2SO_4 (150 ml.) by heating for 3 hours in a stream of nitrogen. After cooling, 2*N* NaOH (190 ml.) was added and the mixture was again heated for ½ hour. The slightly alkaline product was distilled *in vacuo* to remove acyloin and then extracted with ether to remove any neutral resinous matter which might have been formed. 2*N* H_2SO_4 (50 ml.) was then added and the mixture was heated to 100° to convert the acid into lactone. The lactone was extracted with ether and distilled at atmospheric pressure, giving 9 g. of a laevorotatory distillate. The distillation was repeated twice. On the third distillation the lactone showed a constant B.P. of 219° (uncorr.) and the rotation and refractive index were practically unchanged. It had the following properties: $d_{20}^{20°}$ 1·027, $n_D^{20°}$ 1·4393, $[\alpha]_{5461}^{20°}$ −58·11°. The specific rotation was also determined in water $[\alpha]_{5461}^{20°}$ −57·4° (c=2·03). (Found (Weiler): C, 63·06, 62·96; H, 8·72, 8·86%. $C_6H_{10}O_2$ requires C, 63·11; H, 8·83%.)

Oxidation of the lactone. The lactone (1 ml.) was heated with a mixture of conc. HNO_3 (13 ml.) and water (9 ml.) on the water-bath. A violent reaction occurred at first but this soon moderated. In about 2 hours the oxidation was apparently complete. The product was evaporated to dryness *in vacuo*, the crystals were dissolved in water, the solution was made slightly alkaline with ammonia, then acid with acetic acid. Calcium chloride was added to precipitate the oxalic acid. The filtrate on acidification with HCl and extraction with ether (continuous extractor) gave 0·32 g. of crystals which on vacuum sublimation nearly all sublimed giving a product of M.P. 186°. It showed no depression when mixed with succinic acid. The equivalent by titration was 58·6; theoretical for $C_4H_6O_4$ (dibasic) 59·0. Thus succinic acid was produced on oxidation of the lactone with nitric acid. It seems probable therefore that the lactone must have the constitution γγ-dimethyl- or γ-ethyl-butyrolactone, and since it is optically active, only the latter would meet the case.

Racemization and identification of the lactone from terrestric acid. A small sample of *n*-hexanolactone prepared from gluconolactone by heating under reflux with hydrogen iodide and red phosphorus [cf. Kiliani & Kleemann, 1884] was found to be optically inactive. It appeared probable therefore that the lactone from terrestric acid would be racemized by this treatment. This was found to be the case. The optically active lactone (3·7 g.) was heated for 7 hours under reflux with hydrogen iodide (20 ml., sp. gr. 1·7) and red phosphorus (1 g.). The oil-bath was maintained at 150–160°. Water (60 ml.) was added to the cooled mixture which was then extracted with ether (600–700 ml.). The ether was washed with a little water, then with sodium thiosulphate to remove iodine and the acids were transferred to sodium carbonate solution, the lactone remaining in the ether. The aqueous carbonate on acidification gave an oily acid. The ether was removed on the water-bath and the residual lactone was heated for 1 hour on the water-bath with a few g. of granulated zinc and 10 ml. of conc. HCl diluted with an equal volume of water. The product was extracted with ether and treated with sodium carbonate as before. Again a small amount of oil smelling of *n*-hexanoic acid was obtained on acidification of the carbonate solution. This was added to the previous sodium carbonate product. The lactone recovered from the ether was distilled. The B.P. (214°) was probably a little low owing to the small amount recovered. The product was optically inactive and weighed 1·05 g. It was heated in alcohol with phenylhydrazine for conversion into the phenylhydrazide. The product began to crystallize after removal of the alcohol. The crystals were treated with ether, collected and recrystallized from ether. Plates, M.P. 101°. (Found (Weiler): C, 64·93, 64·72; H, 7·93, 8·15; N, 12·49, 12·84%. $C_{12}H_{18}O_2N_2$ requires C, 64·82; H, 8·17; N, 12·61%.) This product was identical with the phenylhydrazide obtained from *n*-hexanolactone prepared by the method of Fittig & Dubois [1890] from dihydrosorbic acid, which also had M.P. 101°. There was no depression in M.P. on mixing the two products.

Treatment of the acidic portion. The acid extracted by sodium carbonate was treated with zinc and HCl exactly as in the case of the lactone in order to remove combined iodine. The product was extracted with ether, washed with sodium thiosulphate and retransferred to sodium carbonate solution. The aqueous solution was acidified and extracted with ether. The product was esterified with diazomethane in ether. The ester after removal of ether was treated with 10 ml. of aqueous ammonia saturated in a freezing mixture and left for several days with occasional shaking. Crystals were obtained; these were collected and weighed 0·27 g. M.P. 99°. After recrystallization from light petroleum the M.P. was raised to 100°. (Found (Weiler): C, 62·78, 62·60; H, 11·09, 10·87; N, 12·54, 12·34%. $C_6H_{13}ON$ requires C, 62·55; H, 11·39; N, 12·16%.)

This substance was *n*-hexanoamide since it showed no depression in M.P. when mixed with synthetic material prepared in the same manner from *n*-hexanoic acid. The synthetic substance crystallized in large plates, M.P. 101°. The formation of *n*-hexanoic acid by reduction of the lactone affords further proof of the constitution of the lactone obtained by hydrolysis of terrestric acid as *n*-hexanolactone.

Bromination of terrestric acid in 50 % aqueous acetic acid.

The conditions were exactly similar to those employed in the bromination of carolic acid [Clutterbuck *et al.* 1935]. Terrestric acid (1·05 g.) dissolved in 50 % aqueous acetic acid (20 ml.) was treated slowly with the theoretical amount (20·1 ml. $\equiv 2Br_2$) of a solution of bromine (2 ml.) in 50 % acetic acid (80 ml.). The product was evaporated in high vacuum over caustic potash. After 4 days' drying the residue (1·57 g.) consisted of crystals and syrup. The crystals were collected and washed with benzene and then with light petroleum. The product (0·53 g.) melted at 169–171° decomp. Further separation occurred in the mother liquor. On recrystallization from benzene the product had M.P. 180° (decomp.), unchanged on sublimation. It gave an immediate strong purple colour with sodium nitrite solution and a red colour with ferric chloride solution and showed no depression of M.P. when mixed with *d*-α-bromo-γ-methyltetronic acid obtained from carolic acid. The M.P. of that acid is stated as 172°, but it was found to melt at the same temperature as the newly isolated product when heated under the same conditions. As the M.P. is really a decomposition point it depends to some extent on the rate of heating. The rotation of the acid in water was determined in a micropolarimeter tube: $[\alpha]^{20^\circ}_{5461} = +8{\cdot}0^\circ$ ($c = 2{\cdot}5$). (Found (Schoeller): C, 31·26; H, 2·79; Br, 41·05 %. Equivalent by titration 196. Bromomethyltetronic acid, $C_5H_5O_3Br$ requires C, 31·10; H, 2·61; Br, 41·40 %. Equivalent (if monobasic) 193.) Since this product is undoubtedly *d*-α-bromo-γ-methyltetronic acid it affords further proof that the acyloin isolated from the products of acid hydrolysis of terrestric acid is actually acetoin.

Thus it has been established that terrestric acid contains a methyltetronic acid nucleus linked to a *l*-γ-hydroxy-*n*-hexanoyl residue. In view of the remarkably close similarity in general properties between terrestric and carolic acids, the constitutions of the two acids must be closely related. The same arguments as developed by Clutterbuck *et al.* [1935] for the constitution of carolic acid may therefore be applied *mutatis mutandis* to terrestric acid, which has thus the constitution of an ethylcarolic acid formulated in the hydrated and anhydrous forms as I and II (p. 2195) respectively.

SUMMARY.

Terrestric acid, $C_{11}H_{14}O_4$, a hitherto undescribed mould metabolic product, is formed on Raulin-Thom glucose medium by three different strains in the *Penicillium terrestre* Jensen series. Its isolation, properties and breakdown products are described. It has been shown to be an ethyl derivative of carolic acid, a metabolic product of *Penicillium Charlesii* G. Smith. Terrestric acid hydrate has been shown to be α-(*l*-γ-hydroxy-*n*-hexanoyl)-*l*-γ-methyltetronic acid.

We wish to thank Mr G. Smith and Mr W. K. Anslow for much technical assistance in the preparation of quantities of crude terrestric acid, and we express our indebtedness to the Research Council of Imperial Chemical Industries, Ltd., for a grant to one of us (J. H. B.).

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CCCVIII. SOME PHYSICO-CHEMICAL CHARACTERISTICS OF THE YELLOW RESPIRATORY ENZYME.

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THE yellow respiratory enzyme isolated by Warburg & Christian [1933] is a substance of some considerable biological interest. By means of a technique involving electrophoresis, Theorell [1935] has recently purified the enzyme and reported its crystallization from solutions containing $(NH_4)_2SO_4$.

This paper is an account of some experiments carried out on a preparation supplied by Dr A. H. Theorell.

EXPERIMENTAL.

The stock material, a yellow paste consisting of enzyme and $(NH_4)_2SO_4$, was stored at approx. 0°.

For experimental purposes solutions of suitable concentration were made by dissolving small quantities of the paste in distilled water and dialysing against distilled water at 2° in cellophane tubes until no sulphate could be detected in the dialysate after 16 hours' contact with the solution.

The early stages of the dialysis were marked by the appearance of a yellow pigment in the dialysate, but this ceased to be detectable some time before a negative SO_4'' test was obtained. In the final stages of the dialysis a slight flocculent brownish precipitate usually formed; this was centrifuged off leaving a golden yellow supernatant solution.

The yellow pigment appearing in the dialysate was shown not to sediment in a centrifugal field of 180,000 times gravity and so was considered to consist of the flavin group derived by decomposition from some of the yellow enzyme, and probably previously associated with the small protein precipitate. Further slight decomposition tended to occur in the solution on standing.

For experiments in the velocity and equilibrium centrifuges quantities of the stock electrolyte-free yellow enzyme solution were diluted with buffers of suitable composition and the resulting solutions used immediately. For electrophoresis and diffusion experiments, however, the solution of yellow enzyme in buffer was dialysed against a large volume of buffer of the same electrolyte composition for 16 hours, to ensure equilibrium.

Sedimentation velocity measurements.

The sedimentation velocity experiments were carried out in a centrifugal field of approximately 180,000 times gravity, the movement of the sedimenting boundary being recorded by the absorption method [Svedberg, 1925; Svedberg & Eriksson, 1932]. A mercury vapour lamp was used as a source of light in conjunction with a nickel oxide filter ($\lambda = 366$ mμ). A single experiment was made using the refractive index method [Lamm, 1928; 1929; McFarlane, 1935; Pedersen, 1936].

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[1] Work carried out during the tenure of a Rockefeller Foundation Fellowship.

The results are given in Table I. In every case sufficient electrolyte was present in the solutions to suppress any charge effect on the sedimentation.

Table I. *The sedimentation constant of the yellow respiratory enzyme.*

Exp. no.	Buffer	Total salt conc. (M)	pH	$S_{20} \times 10^{13}$
15	HOAc, NaOAc, NaCl	0·180	4·46	5·94*†
13	HOAc, NaOAc, NaCl	0·180	4·95	5·79
10	KH_2PO_4, Na_2HPO_4, NaCl	0·300	5·72	5·72
17	KH_2PO_4, $Na_2B_4O_7$, NaCl	0·174	5·84	5·73
7	KH_2PO_4, Na_2HPO_4, NaCl	0·150	6·00	5·80
11	KH_2PO_4, Na_2HPO_4, NaCl	0·129	6·71	5·60
21	KH_2PO_4, Na_2HPO_4, NaCl	0·180	7·31	5·81
8	KH_2PO_4, Na_2HPO_4, NaCl	0·150	8·00	5·68
25	KH_2PO_4, $Na_2B_4O_7$, NaCl	0·150	8·65	5·82
27	$Na_2B_4O_7$, Na_2CO_3, NaCl	0·240	9·11	5·70
24	$Na_2B_4O_7$, Na_2CO_3, NaCl	0·240	9·65	5·93
26	$Na_2B_4O_7$, Na_2CO_3, NaCl	0·240	10·09	5·57†
22	KH_2PO_4, Na_2HPO_4, NaCl	0·242	11·48	5·63*†
19	HOAc, NaOAc, NaCl	0·183	4·46	6·14*†
20	Same solution as 19, but 24 hours later	—	--	6·38*†

Mean value of $S_{20} = 5·76 \pm 0·09 \times 10^{-13}$.

* Some decomposition. † Omitted in calculating mean.

From the data of Table I it may be seen that the sedimentation constant, S_{20}, remains substantially constant over the pH range 5 to 9·5, which may be identified with the stability region of the enzyme. Below pH 4·5 there is a tendency for the enzyme to aggregate and for the flavin group to be liberated. At pH 4 this liberation occurs so rapidly that no sedimentation can be observed using light of wave-length 366 mμ.

The sedimentation constant $5·76 \pm 0·09 \times 10^{-13}$ is the mean of the values of S_{20} over the stability region.

In the case of Exp. 10, which was carried out by the refractive index method, the refractive increment due to the enzyme contained in the solution remained constant during the whole experiment. Moreover, the "spreading coefficient" showed no drift with time [McFarlane, 1935]. These two facts indicate that the preparation investigated was monodisperse, and this conclusion is supported by the curves obtained when the light absorption method was used.

The diffusion coefficient.[1]

The diffusion coefficient was determined by the method of Lamm & Polson [1936] and the data were analysed by means of equation (2) of their paper and by comparison of the experimental curve with the normal dispersion curve. The data follow:

Buffer: 0·070 M KH_2PO_4, 0·010 M Na_2HPO_4, 0·1 M NaCl; pH = 5·86.

Time of diffusion hours	D cm.^{2} × 10^{-7}/sec.
17	6·07
27	6·01
41	6·13
Mean	6·07

$D_{\text{corr.}} = 6·28 \times 10^{-7}$ cm.^{2}/sec.

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[1] We are indebted to Mr A. G. Polson for carrying out this measurement.

The value of $D_{\text{corr.}}$ is for pure water at 20°, the correction being made by equation (10) of Lamm & Polson [1936].

Comparison with the normal dispersion curve showed the substance to be monodisperse, which is also indicated by the absence of drift in the diffusion constant with time.

The partial specific volume.[1]

The partial specific volume occurs as a term in the equations used for calculating molecular weight from sedimentation data and was accordingly determined.

The determination was made on an electrolyte-free aqueous solution of the yellow enzyme at 20°. The value obtained was 0·731, which is low in comparison with most proteins, the usual range being 0·745–0·750.

The molecular weight.

It has been shown [Svedberg, 1925; 1927] that the molecular weight of a substance can be calculated from sedimentation and diffusion data by means of the equation

$$M=\frac{RTs}{D(1-V\rho)}.$$

By substituting the values previously reported in this paper into the above equation, a value of 82,800 is obtained for the molecular weight of the yellow enzyme.

The molecular weight was also determined by sedimentation equilibrium, using the refractive index method [Lamm, 1929; Pedersen, 1936]. Owing to the small amount of material available, the refractive increment due to the enzyme in the solution used for the equilibrium experiment was estimated by carrying out a refractive index velocity experiment on the same solution. The molecular weight was then calculated from equations (12) and (15) of Pedersen's paper.

The actual determination was carried out at 20° using a phosphate buffer, *p*H 5·72. Two cells at different distances from the axis of rotation were used, and the equilibrium was measured for two speeds of rotation.

In Table II the data for one cell (cell A), at 120·9 rev./sec., is given in full; the remaining results are mean values. For the significance of the symbols see Pedersen [1936].

It will be observed that there is no drift in the M_{wx} values with distance from the centre of rotation. The M_{zx} values fluctuate considerably, and the observed molecular weight tends to fall as distance from the centre of rotation increases, which is the opposite of the behaviour expected for a polydisperse preparation. In general the data indicate that the substance is monodisperse.

The mean of all values obtained for the molecular weight from sedimentation equilibrium data is 77,500, which is rather lower than that calculated from the sedimentation and diffusion constants.

The mean of the values from sedimentation velocity and diffusion data and sedimentation equilibrium measurements is 80,000.

By means of a photoelectric determination of the flavin content of the yellow enzyme Theorell [1935] has estimated the molecular weight, on the assumption that the enzyme contains one flavin group in the molecule. The values obtained for several preparations vary from 70,000 to 75,000; these are in fairly good agreement with the figure just given.

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[1] We are indebted to Dr A. H. Theorell for carrying out this determination.

Table II. *The molecular weight of the yellow respiratory enzyme by sedimentation equilibrium.*

Cell A; 120·9 rev./sec.

x	$Z\ \mu$	$dn/dx.10^3$	$(n_1 - n_0).10^3$	M_{wx}	M_{zx}
4·80	415	4·409	2·074	74,200	
					84,900
4·85	474	5·036	2·310	75,300	
					79,500
4·90	538	5·716	2·579	75,800	
					77,600
4·95	609	6·471	2·883	76,000	
					74,800
5·00	688	7·310	3·227	75,900	
					76,100
5·05	779	8·277	3·616	75,900	
					74,700
5·10	881	9·360	4·057	75,800	
			Mean	(M_w) 75,600	(M_z) 77,800

Cell	Rev./sec.	M_w	M_z
A	120·9	75,600	77,800
A	145·1	(64,800)	77,800
B	120·9	82,800	77,000
B	145·1	74,400	76,600
		Final mean 77,500	

Electrophoretic mobility measurements.

A series of measurements of the electrophoretic mobility of the yellow enzyme was made, using the method of Tiselius [1930; *v.* also Pedersen, 1933], with the same recording conditions as for the sedimentation velocity experiments.

Measurements were made in acetate buffers (0·02 M NaOAc + xM HOAc), and phosphate buffers of constant ionic strength ($\mu = 0{\cdot}02$), all the experiments being carried out at 20·0°.

Electrophoretic mobility of the yellow enzyme.

pH	Buffer	Mobility (u) cm./sec.. 10^{-5}	Direction
4·60	Acetate	4·73	Cathodic
4·87	,,	2·37	,,
5·45	,,	1·14	Anodic
5·41	Phosphate	1·53	,,
6·14	,,	4·72	,,
7·45	,,	8·06	,,

Isoelectric point pH 5·22; slope of the mobility curve $\frac{du}{d\,(p\mathrm{H})} = 6{\cdot}4 \times 10^{-5}$ at the isoelectric point.

The curves obtained showed no signs of inhomogeneity, and the value for the isoelectric point is in good agreement with that found by Theorell [1935], viz. 5·25, using another method.

DISCUSSION.

From a consideration of the data which have been presented it may be seen that the yellow enzyme preparation studied was monodisperse and electrochemically homogeneous. It therefore seems reasonable to conclude that the substance is a chemical entity of molecular weight 80,000.

The agreement between the molecular weight determination from ultracentrifugal data and by estimation of the flavin content of the molecule indicates that the molecule contains one flavin group.

SUMMARY.

1. Sedimentation, diffusion and electrophoresis data are presented for the yellow respiratory enzyme.

2. On the basis of the experimental results it is concluded that the preparation studied was a chemical entity of molecular weight 80,000.

3. The data indicate that the yellow enzyme contains one flavin group in the molecule.

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CCCIX. PYRUVIC ACID OXIDATION IN BRAIN.

I. VITAMIN B_1 AND THE PYRUVATE OXIDASE IN PIGEON'S BRAIN.

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THE object of the present paper and others to follow is to present the evidence for the pyruvate oxidase theory of the action of vitamin B_1. In brain tissue treated with lactate, we now believe that oxidation mainly follows the expected sequence

$$\text{Lactate}+\text{O}\xrightarrow{\text{A}}\text{pyruvate}+(n)\text{O}\underset{\underset{\text{vit. }B_1}{\uparrow}}{\xrightarrow{\text{B}}}\text{oxidation products}\quad ......(1),$$

and that the action of the vitamin is exerted at stage B.[1] In so exerting an effect, which can be observed noticeably only in the avitaminous brain (or kidney),[2] this vitamin is either acting as a coenzyme to pyruvate oxidase, or constitutes the prosthetic group of the enzyme itself. At present evidence does not decide between these possibilities, though favouring the latter.

At first, in this laboratory, we favoured the lactate oxidase theory of the action of vitamin B_1 [Kinnersley & Peters, 1930], based upon the large accumulations of lactic acid, which are a feature of vitamin B_1 deficiency. Later this view was discarded; especially we may mention that Meiklejohn [1933] did not find an increased disappearance of lactic acid associated with increased oxygen uptake in presence of the vitamin. There came subsequently the discovery [Peters & Sinclair, 1933] of a substance giving the colour reaction of pyruvic acid as an abnormal product in avitaminous brain respiring in lactate, the subsequent study and identification of this [Peters & Thompson, 1934], and finally the finding of abnormal amounts of pyruvic acid in avitaminous blood [Thompson & Johnson, 1935; Johnson, 1936]. From the time of this work, there has always been a tempting body of evidence in favour of the importance of pyruvate (as an intermediate) in brain respiration systems, and of the close correlation if not identification of vitamin B_1 with the pyruvate oxidation system. The evidence of abnormal amounts of pyruvate in blood was important as it indicated that the phenomenon shown *in vitro* by teased brain also took place *in vivo*, a complete answer to any criticism of the value of such *in vitro* work. In spite of these indications, there seemed to be certain facts which could not be reconciled with the direct pyruvic oxidase view; this led us to hesitate in its adoption. As the majority of these have now been removed, there remain no outstanding objections to the pyruvic oxidase theory. We can now consider some of the objections in detail; the attempt to answer them has brought to light a few new facts about brain enzyme systems.

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[1] This scheme was presented by the author [1936], and with McGowan [1936].

[2] The catatorulin effect is shown by pigeon's kidney [Thompson, 1934].

The relation of lactate and pyruvate.

It is natural of course to believe that respiration in brain tissue follows the sequence (1). If this is so, and if the vitamin acts at stage B, then we should expect (*a*) the accumulation of pyruvate from lactate in absence of vitamin B_1 as is now known to occur; (*b*) less O_2 uptake with pyruvate than lactate in the avitaminous brain, and the reverse with normal brain, because in the latter we might expect that conversion of lactate into pyruvate was not sufficiently rapid to saturate the pyruvate oxidase with pyruvic acid; (*c*) a more pronounced catatorulin effect (vitamin B_1 extra O_2 uptake) with the pyruvate than with lactate. In early work upon this point, Meiklejohn *et al.* [1932] found that (*b*) occurred, but that (*c*) did not apply; there were cases in which brain tissue apparently reacted with vitamin B_1 in presence of lactate and not of pyruvate. This seemed to be irreconcilable with scheme (1). Let us first consider work upon (*c*).

Vitamin B_1 effect and pyruvate. In the last two years a very large number of catatorulin and other tests have been done with pyruvate. These are summarized separately. In over 50 consecutive experiments, there has been no case in which large vitamin effects have not been shown with this substrate. On the other hand, I have occasionally noticed failures with lactate. This is consistent with scheme (1), and not with the early pyruvate work, and suggests that the early failures were due to some special feature of the experiments. In a footnote, Peters & Thompson [1934] considered that the action of pyruvate at *p*H 7·3 must be linked with that of pyrophosphate (as was lactate at *p*H 6·6 [Peters & Sinclair, 1933]). This was because it seemed to be a possible difference between the earlier and later experiments. I have now submitted this point to direct tests, using even the more alkaline *p*H 7·7 in order to emphasize any differences, and can obtain no support for this opinion. In the experiments of Table I, at *p*H 7·3, Exp. 965 (one of two similar ones with normal brain) is confirmatory of previous work by Meiklejohn *et al.* [1932] who worked without pyrophosphate. There is a larger uptake for normal brain with pyruvate than with lactate in complete agreement with postulate (*b*). The significant vitamin effect in pyruvate, Exp. 965, is unusual, but it is interesting that it happens to occur with pyruvate; the slight inhibition with vitamin and lactate observed in 1935 has often been noticed in the normal brain and is not yet explained. At this *p*H of the avitaminous brain the lower O_2 uptake with pyruvate than with lactate is reproducible (Exp. 967). Even with pyruvate plus vitamin there is a higher total oxygen uptake than with the lactate, contrary to the normal. Nevertheless, the other feature of the earlier experiments is not reproduced: there is a marked catatorulin effect with pyruvate, even larger than that with lactate. At *p*H 7·7 (Table I (*b*)) the relations with lactate and pyruvate in the five experiments quoted are substantially the same. There is always a catatorulin effect with pyruvate.

There is if anything a greater restoration with pyruvate than with the lactate.

The relations to pyrophosphate at this *p*H are perplexing. There is no evidence from Exps. 959 and 956 that it is necessary for respiration with pyruvate. It is in fact inhibitory in these experiments both to lactate and to pyruvate. The inhibition to pyruvate persists right through the period of respiration though with diminishing intensity. That for lactate is over (as in the case of the *p*H 6·6 respiration) (see below) after about 1½ hours.

For the moment it is not possible to explain all these points, but it is clear that they do not support the explanation of the older experiments of Meiklejohn *et al.*, who reported occasional failure to obtain the vitamin effect with pyruvate.

Table I. *Comparison of O_2 uptakes with pyruvate and lactate as substrates at varying* pH *values.* (*Normal and avitaminous pigeons' brains.*)

µl./g./hr. P = pyruvate; L = lactate; V = vitamin; pp = pyrophosphate; Res = residual.

(*a*) *p*H 7·3. Cool preparation (14°).

Exp. 965. Normal cerebrum.

							Vitamin difference	
Period (min.) ...	15	15	30	30	30	30	0–60	60–120
P	2740	2460	2110	1745	1410	1210	+270	+225
PV	3035	2675	2390	2000	1600	1435		
L	2810	2465	1995	1460	1070	855	− 82	− 30
LV	2650	2350	1945	1450	1020	850		
Res	1010	935	870	595	376	300		

Experiment quite exceptional in size of the vitamin effect shown with pyruvate by the norma brain.

Exp. 967. Avitaminous cerebrum.

P	1170	1040	660	545	415	367	+458	+530
PV	1480	1425	1225	1080	945	837		
L	(1405)	1335	1055	860	640	525	+350	+375
LV	1630	1650	1485	1235	1010	965		

(*b*) *p*H 7·7. All experiments avitaminous. Exps. 957, 961, 959 prepared "cool", remainder "warm".

Exp. 957.

							Vitamin difference	
Period (min.) ...	15	15	30	30	30	30		
Res	1270	1000	785	540	350	330	+124	+100
ResV	1250	1090	900	610	480	390		
P	1840	1435	1120	750	551	370	+470	+600
PV	2090	1945	1680	1315	1140	914		
L	1870	1705	1330	976	742	519	+664	+650
LV	2535	2305	2050	1625	1395	1100		

L, LV, single estimations.

Exp. 961.

Cerebrum.								
P	2120	— 1520	—	1090	745	490	+410	+710
PV	2355	— 2110	—	1815	1435	1130		
L	2100	— 1775	—	1330	1010	700	+505	+590
LV	2550	— 2335	—	1950	1575	1225		
Optic lobes, rest.								
P	1590	— 1150	—	1070	710	485	+390	+480
PV	1910	— 1610	—	1485	1250	1060		

Exp. 959.

P	2190	1865	1260	945	750	—	+518	+595
PV	2570	2280	1900	1620	1265	—		
Ppp	2040	1670	1210	940	620	—	+223	+365
PppV	2105	1540	1540	1255	1035	—		
L	2640	2680	1830	1380	1040	—	+114	+347
LV	2625	2410	2200	1740	1375	—		

L, LV, single estimations.

Exp. 958.

							Vitamin difference	
Period (min.) ...	15	15	30	30	30	30		
P	— 1900	—	1200	900	624	—	+825	+838
PV	— 2670	—	2100	1760	1425	—		
L	— 2210	—	1645	1115	895	—	+588	+537
LV	— 2720	—	2310	1725	1360	—		
Lpp	— 2170	—	1590	1200	986	—	+190	+465
LppV	— 2290	—	1850	1725	1390	—		

P, PV, single estimations.

Exp. 956.

P	2260	1635	1160	775	470	440	+756	+905
PV	2760	2415	2032	1770	1280	1080		
Ppp	1970	1550	1180	940	560	395	+472	+490
PppV	(2350)	2110	1655	1320	1160	930		

How are the results of the previous work to be explained? There is no difference in the time of year at which the experiments were carried out. The older work was done with brain minced upon a warm plate at room temperature, which might have been rather low upon occasion. I have therefore repeated four such experiments, and quote two, Table II (*a*) and (*b*), Exp. 971. In all cases a good pyruvate effect was found. In Exp. 970, there was used the crude preparation of vitamin B_1 which had been previously employed by Meiklejohn *et al.* in their work. It had been stored for 3 years without change so far as could be ascertained. There was no evidence of any toxic effect either in this or in other experiments. We can therefore exclude the vitamin and also the conditions of mincing. This leaves us with possible variations in the medium, either in the inorganic constituents or in the pyruvate itself. Previously we had employed a Ringer solution which originally contained calcium. After bringing to *p*H 7·3 approximately, the precipitated Ca phosphate was filtered off. Later we used Ringer without calcium. We had not thought that there would be much difference in view of the smaller amounts of Ca involved, though Sinclair had found that presence of Ca produced slight inhibition. Further tests showed that the addition of traces of Ca make a difference, see Table II (*c*), but this is not enough

Table II. *Effect of cool preparation and other changes.*

*p*H 7·3. Pyruvate as substrate always present.

(*a*) Increasing amounts of pyruvate (cool preparation).

Exp. 968. Normal cerebrum.

						Vitamin difference	
Period (min.)...	15	15	30	30	30	0–60	60–120
Pyr 0·023 *M*	3065	—	2505	2230	1780	—	—
Pyr 0·046 *M*	3095	—	2595	2235	1750	—	—
Pyr 0·069 *M*	2890	—	2680	2195	1670	Slight inhibition	

(*b*) Pyruvate: acid neutralized without dilution (WP).

Exp. 971. Avitaminous cerebrum (cool preparation).

P	1650	1050	800	616	508	+550	+580
PV	2030	1650	1405	1240	1040		
WP	1352	1090	785	610	500	+450	+410
WPV	1725	1550	1250	1070	850		

(*c*) Effect of calcium addition. Exp. 1, 0·015 *M*; Exp. 2, 0·0003 *M* $CaCl_2$.

Exp. 1. Avitaminous.

P	1410	1085	815	576	—	+580	564 (30 min.)
PV	1860	1630	1475	1140	—		
CaP	1105	870	745	560	—	+425	540 (30 min.)
CaPV	(1450)	1275	1220	1100	—		

Exp. 2. Avitaminous.

P	2300	1710	1355	972	765	+610	+670
PV	2710	2390	2035	1720	1360		
CaP	1990	1600	1250	1010	790	+530	+605
CaPV	2370	2090	1880	1650	1457		

(*d*) Comparison of crystalline vitamin (4γ) with old vitamin concentrate (46).

Exp. 970. Avitaminous.

P	— 990	—	698	505	372	+525	+500
PV	— 1500	—	1210	968	810		
P	— 960	—	655	480	373	+512	+450
P (46)	— 1430	—	1235	1020	835		

to remove the catatorulin effect with pyruvate.[1] This leaves us with only one possibility, the pyruvate itself. As was stated at the time, the pyruvic acid was highly purified by distillation and though not crystallized was considered to be pure enough for the purpose. But we did not realize then the profound change which can be made in pyruvic acid by neutralizing direct with N alkali before previous dilution with water. Peters & Thompson [1934] found that upon occasion a large change in bisulphite-binding power might rapidly occur. In the early experiments, in order to save volume, 80–100 mg. of purified pyruvic acid were weighed out into a tube and the alkali added direct. This seems to be dangerous, though the conditions under which the change occurs have not been worked out. In the experiment in Table II (*b*) the vitamin effect is reduced with pyruvic acid neutralized before dilution. With smaller amounts of pyruvate the reduction in pyruvate itself might be quite sufficient to account for the results. The early experiments lasted only for 1 hour: if continued they might even then have shown an effect. I think that they can be safely set aside as due to unrecognized experimental error. The present pyruvate gives only slight inhibition at 0·067 M (Table II (*a*)).

If the view is correct that the vitamin action is upon the pyruvate only, it should be possible to find that, in the early stages of an experiment, there is a larger catatorulin effect with pyruvate than with lactate. At a later stage, when sufficient pyruvate is present, formed from lactate, there should be no difference. A decisive experiment of this kind has proved difficult to make; it will clearly be much governed by details such as the rate of diffusion of either the pyruvate or lactate to the active centres concerned. By keeping for some time at the low temperature diffusion effects will be minimized, but there will be a possibility of sufficient action of the lactate oxidase even in the initial 12 min. required for equilibrium at 38°. Some trials of this point proved rather inconclusive, though there was a balance in favour of slightly increased catatorulin effect in the first half-hour only with pyruvate.

The evidence of the delayed substrate experiment.

As the result of delayed substrate experiments (Quastel and Wheatley technique), Peters *et al.* [1935] advanced the following view, considering respiration as a chain of reactions.

$$\underset{\substack{\uparrow\\ \text{vit. B}_1\\ \text{pyrophosphate}}}{X} \longrightarrow Y + \begin{matrix}\text{Pyruvate or}\\ \text{Lactate}\end{matrix} = \begin{matrix}\text{Products of oxidation and}\\ \text{oxidative synthesis}\end{matrix} \qquad \ldots\ldots(2).$$

This hypothesis was an attempt to explain the following facts. With tissue suspended in Ringer phosphate only, with and without addition of vitamin, the increase upon addition of substrate was greater for tissue previously incubated with vitamin and greater still for vitamin plus pyrophosphate. These facts have been confirmed recently, but at the same time other findings have led to the substitution for (2) of the alternative interpretation, first proposed by the same authors in a footnote. According to the latter, the facts observed in this type of experiment are due to the protection of the essential factor from irreversible destruction. This interpretation now supports instead of opposing the pyruvic oxidase theory.

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[1] The effect of Ca is related probably to the colloidal ion effect, see Ashford & Dixon [1935], Dickens & Greville [1935].

The sequence lactate-pyruvate at acid reactions.

I was led to revise views upon hypothesis (2) by fruitless attempts to confirm it directly (with Passmore), and by experiments with the delayed technique, at acid reaction. Peters & Sinclair [1933] had shown that at *p*H 6·6 (instead of the normal *p*H 7·3) there was no catatorulin effect with lactate in absence of pyrophosphate, whereas with pyruvate an effect was found. They had however been unable to demonstrate formation of pyruvic acid by colour reaction; this had been another point irreconcilable at the time with the pyruvic oxidase theory. In the more recent and similar Exp. 861, Table III, made under more modern conditions, we see that with the "warm" minced tissue there was a slight catatorulin effect with lactate without pyrophosphate. With lactate + pyrophosphate, the vitamin effect as also the respiration is slightly reduced at first, but later increased over that of lactate alone. With pyruvate alone a large difference was present. The experiment is therefore in the same sense as the earliest ones, but the loss of vitamin effect in lactate is not so pronounced.

Table III.

Acid Ringer phosphate *p*H 6·4. Lactate and pyrophosphate.

Exp. 861. Warm preparation. Avitaminous.

Period (min.) ...	0–15	15–30	30–60	60–80	Vitamin differences			
L	1480	940	650	430	170	160	140	134
LV	1600	1100	790	564				
Lpp	1260	1010	750	570	80	130	260	330
LppV	1340	1140	1010	900				
P	1310	950	595	365	250	300	415	435
P + V	1560	1250	1010	800				

Exp. 866. Warm preparation. Normal brain.

Period (min.) ...	0–15	15–45	45–75	75–105	105–135
(1) O	1530	1170	708	537	400
(2) L 0·041 *M*	1580	1440	1030	725	615
(3) L 0·082 *M*	1160	1270	1115	775	627
(4) L 0·164 *M*	865	1035	800	740	526
(5) Lpp 0·041 *M*	1430	1340	1080	**1020**	**918**
(6) Lpp 0·082 *M*	1150	1000	875	815	675
(7) Lpp 0·064 *M*	755	755	615	514	527

Designed to explore the optimum concentration of lactate at this *p*H and the relation to pyrophosphate.

Exp. 863. Warm preparation. Normal brain.

Period (min.) ...	15–45	45–75	75–105	105–135
Nil	1090	576	422	400
L 0·0035 *M*	1520	1040	785	615
L 0·015 *M*	1082	925	700	580
Pyr 0·017 *M*	1800	1570	1330	1142

Initial reduction of respiration in lactate at *p*H 7·3 by pyrophosphate has now been well established by Johnson [1936].

Exp. 866 (Table III) is a complete experiment upon normal tissue and shows again the inhibition with pyrophosphate which is present for 1¼ hours only. After this the pyrophosphate has exerted a marked improvement upon the "lactate" respiration. Inhibition of the respiration, especially during the initial periods, is seen with increasing concentration of lactate alone, but this becomes much more marked when pyrophosphate is present; compare for instance L and Lpp (0·164 *M*). In the latter case, with the action of pyrophosphate the increase of O_2 uptake over the residual has been entirely eliminated. The lactate at this *p*H (with the normal brain) does not nearly reach the level of pyruvate respiration, as is shown by Exp. 863. Note again that even at a higher concentration of lactate there is inhibition only for $1\frac{3}{4}$ hours. It is clear that the relations with pyrophosphate are complex.

The above facts are consistent with the view that the substrate also at this *p*H producing the largest respiratory effect is pyruvate. Exp. 866 is explained if pyrophosphate is helping the conversion of lactate into pyruvate.

It remains to be proved that pyruvic acid is formed at *p*H 6·6. Table IV gives the few experiments made upon this point. With normal brain (two experiments) there was a slight formation of pyruvate (B. B. S.) from lactate only (Exps. 995, 996); this was reduced by pyrophosphate, which increased O_2 uptake in 2 hours, but was again increased by adding iodoacetic acid. In the one experiment tried, there was more pyruvate formed in presence of pyrophosphate. With the avitaminous brain there was less pyruvic acid present with the pyrophosphate in spite of the increased O_2. The few results justify the conclusion that pyruvic acid is formed, though in lower amounts than at *p*H 7·3; but there is no experimental support for the suggestion that pyrophosphate improves the yield of pyruvate; actually it was decreased.

Table IV. *Formation of pyruvate at* p*H 6·4.*

Brain tissue allowed to respire in presence of L/pyrophosphate, V or iodoacetate, 0·1 mg./ml. for 2 hours; after cooling 1 ml. of 25 % trichloroacetic acid added, filtered and bottles washed with 1 ml. + 1 ml. 5 % trichloroacetic acid. Samples (2 ml.) analysed for bisulphite-binding substances (Clift & Cook) or by 2:4-dinitrophenylhydrazine. (Case as modified by Peters & Thompson.)

Figures are mg. pyruvic acid/g. tissue formed in 2 hours, and are means from duplicate bottles estimated separately, except in 1146 where the two bottles were mixed and so estimated.

							Cool preparation	
Exp.	Condition	L	LV	Lpp	LppV	LIAA	LIAApp	Method
995	Normal	0·9	—	0·53	—	—	1·24	Bisulphite-binding (BBS). Duplicate poor
996	,,	0·51	—	0·33	—	0·47	0·81	,,
998	Avitaminous	1·66	1·16	2·00	1·10	—	—	,,
1146	,,	1·42	0·45	1·17	0·35	—	—	Hydrazone
1153	,,	0·91	—	0·38	—	1·67	1·21	BBS

Interpretation of the delayed substrate experiments.

At first sight these experiments are much more difficult to reconcile with the view that the sequence of oxidation is lactate→pyruvate. By working at *p*H 6·6, however, it is possible to dissect the reactions better.

Exp. 863 (Table V) shows the extent of variations in the initial respiration which may be expected to be due to experimental error and that addition of vitamin to the normal has no influence, a now well-established fact; again in the delayed substrate experiment there is a much larger respiration with pyruvate than with lactate at this *p*H.

The effect of pyrophosphate (Exp. 878) in the initial stages appears to provide a much clearer argument for the scheme proposed by Peters *et al.* even than before. In absence of the substrate, respiration with and without pyrophosphate

Table V. *Delayed substrate.*

Normal cerebrum. pH 6·6. Warm preparation.
Additions during course of experiment at arrow ↓.

O = Ringer phosphate alone; pp = pyrophosphate; P = pyruvate; L = lactate. Addition at arrow made to bottles after removal from the bath. Bottles refilled with O_2 and returned to bath. Single estimations.

Exp. 863.

Period (min.)...	30	30	30	↓	20	25	36	30
O	2220	1575	960	LV	765	905	725	602
V	2020	1475	920	L	800	815	645	512
O	2130	1485	925	PV	940	1115	970	850
V	2060	1462	932	P	1030	1095	985	860

Exp. 878.

Period (min.)	30	30	30	↓	15	15	20	15	30	30
O	1685	1180	730	Ppp	674	800	665	515	585	460
pp	1370	940	650	P	1280	1330	1330	1215	1270	1170

Period (min.)	30	30	30	30	30	↓	15	15	15	15	15	30	15
O	1450	1040	754	480	360	Ppp	290	195	70	218	140	140	175
pp	1310	855	674	440	370	P	870	880	912	962	1070	960	960

reaches approximately the same level, but upon addition of the substrate the difference in respiration indicates the extent of the underlying difference in the state of the tissue.

The longer the addition of the substrate is delayed (Exp. 878, Table V) the more marked is the effect, even after an initial period of 1½ hours in which respiration of O and pp have been identical within experimental error for at least an hour. The reaction of the pyruvate oxidase is not a function of the respiratory state of the tissue, but of some reaction here improved by pyrophosphate.

The next experiment (862, Table VI) adds the vitamin effect but introduces a new point. Without substrate, we see a slight effect of vitamin. When substrate is added after 1¾ hours, pyruvate is more effective than lactate. Lactate in the initial period without addition of pyrophosphate was inhibitory, in this case even with vitamin. Subsequent addition of pyruvate was not effective in giving a large respiration in the last hours unless pyrophosphate was present.

In Exp. 1161 again lactate is less effective than pyruvate in restoring respiration after some 2 hours. This suggests that some part of the lactate oxidase system has broken down in this time. Further, some part of the system is better maintained in the presence of lactate, pyrophosphate and vitamin, viz. that which interacts with pyruvate.

For stabilization of pyruvate oxidase we do not need to use large amounts of lactate. In Exps. 896 and 897 the chief points which need comment are that 1 mg. of lactate in the initial period added with pyrophosphate gives a low respiration compared with pyruvate after 1½ hours of respiration, but maintains the pyruvate oxidase system intact. This can be seen by the recovery to the value given by pyruvate alone in the initial period upon adding pyruvate as a delayed substrate. For demonstration of this maintenance 0·5 mg. of lactate upon occasion has proved to be effective for a period up to 2 hours (Exp. 899), the action of lactate in this respect requiring pyrophosphate and vitamin. In other

Table VI. *Avitaminous and normal brain. Delayed substrate. Vitamin and effect of small amounts of lactate upon stability of pyruvate.*

*p*H 6·4. Warm preparation.
Additions at arrows.

Exp. 862. Avitaminous (single estimations).

Period (min.) ...	30	30	30	30	26 ↓	15	30	30
O	1400	1200	1095	795	LV	630	407	365
OV	1520	1440	1135	895	L	765	585	506
O	1490	1310	960	685	PV	585	464	460
OV	1600	1520	1195	950	P	945	780	700
L	965	— 660 —		543	PV	755	632	610
LV	1455	— 1155 —		795	P	655	504	467
Lpp	1160	875	805	705	PV	495	412	370
Lpp, V	1320	1170	1060	990	P	1045	985	878

Exp. 1161. Avitaminous.

Period (min.) ...	15	23	29	↓	60
pp	1235	950	780	PV	798
ppV	1680	1244	1075	P	1362
pp	(1275)	(990)	750	LV	880
ppV	(1545)	(1200)	(1120)	L	1182

Exp. 896. Normal cerebrum.

Period (min.) ...	15	45	30	30	30 ↓	15	20	20	20
pp	1400	995	640	455	Pyr	980	1060	1030	990
Pyr pp	2730	2460	2150	1950		1700	1550	1460	1350
L 0·0037 *M* pp	2335	1980	1720	1480	Pyr	1810	1680	1620	1560
L 0·0055 *M* pp	(2170)	(1950)	(1700)	(1550)	Pyr	1680	1615	1540	1500

Exp. 897. Normal cerebrum.

Period (min.) ...	30	30	30	30	30 ↓	15	15	20	20	20
Pyr 0·015 *M* pp	2375	2180	2050	1855	Pyr	1630	1500	1370	1300	1205
Pyr 0·015 *M*, L 0·0037 *M* pp	2275	2050	1900	1760	Pyr	1550	1415	1265	1250	1120
L 0·0037 *M* pp	1955	1645	1575	1360	Pyr	1655	1520	1475	1290	1225
L 0·011 *M* pp	1915	1760	1610	1540	Pyr	1610	1580	1460	1300	1300

Pyruvate at arrow added to total concentration of 0·023 *M*.

Exp. 899. Avitaminous cerebrum.

Period (min.) ...	30	30	30	30	30 ↓	15	15	30	30
L 0·0018 *M* pp	1420	1125	980	840	PyrV	850	790	760	800
LppV	1730	1385	1215	1080	Pyr	1275	1300	1140	1110

Table VII. *Pyruvate and pyrophosphate.*

Exp. 871. Normal brain; *p*H 6·4; warm preparation.

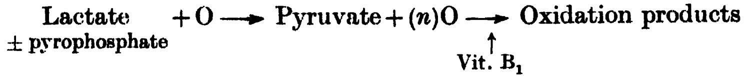
Period (min.) ...	0–15	15–45	45–75	75–105	105–120
O	1170	1180	840	750	355
pp	1195	910	672	625	390
Pyr 0·016 *M*	2055	1910	1870	1780	1535
Pyr 0·016 *M* pp	2200	1990	1860	1753	1625
Pyr 0·032 *M*	1925	1780	1660	1485	1335
Pyr 0·032 *M* pp	2010	1890	1645	1510	1290

experiments it has been found that at this pH pyrophosphate has very little influence upon the respiration of pyruvate, see Exp. 871, Table VII.

There is inhibition here with excess of pyruvate but the pyrophosphate has very little influence upon O_2 uptake. The essential point at this pH then is the linkage of pyrophosphate and lactate and the independence of pyruvate and pyrophosphate. In absence of pyruvate, lactate and pyrophosphate together with vitamin are essential for the maintenance of the stability of the pyruvate oxidase system.

The experiments given in Table V have been selected from a large number of attempts to decide these points, and I regard them as significant. In general I have reached the conclusion that the delayed substrate experiment is so much affected by problems of diffusion that it is not a valuable technique. It will be clear that the demonstration of these discontinuities between the lactate and pyruvate systems requires rather fine adjustment. The pyruvate oxidase is very unstable in absence of either pyruvate or vitamin at 38°, hence the decisive point in the experiment may often be missed. The peculiar details of the survival respiration in these mixed enzyme systems indicate again that the most important component is pyruvate. In absence of either lactate plus pyrophosphate or of pyruvate itself, the pyruvate oxidase system appears unstable; rapidly disappearing at 38°.

Further it is clear that some component of the lactate oxidase system is also unstable at this pH; this might be the coenzyme in view of recent work by Green & Brosteaux [1936] but this needs proof. Another important component might be lactoflavin; hence the importance of preparing specimens under dark conditions, as has of late been uniformly our practice. The picture fits scheme (1) modified as (1a), but not the old scheme (2).



$$\underset{\pm\text{ pyrophosphate}}{\text{Lactate}} + \text{O} \longrightarrow \text{Pyruvate} + (n)\text{O} \underset{\underset{\text{Vit. B}_1}{\uparrow}}{\longrightarrow} \text{Oxidation products} \quad ......(1a)$$

provided we assume that the pyruvate oxidase is unstable in absence of pyruvate or of vitamin B_1.

Both lines of evidence now strengthen the pyruvate oxidase theory instead of being opposed to it. There still remain some unreconciled data. (a) Peters & Thompson [1934] could find no consistency in the ratio $\frac{\text{Pyruvate disappearing}}{\text{Extra oxygen uptake}}$ either at pH 7·3 or 6·6. Further, the vitamin effect was larger with lactate than with pyruvate; this is not immediately to be reconciled with the action of vitamin B_1 only on the pyruvate either preformed or arising from lactate. (b) Peters *et al.* [1935] found that fluoride inhibited the appearance of pyruvate in presence of lactate, as well as inhibiting the O_2 uptake with pyruvate; but fluoride has not been supposed to stop the action of lactate oxidase. (c) The failure to increase pyruvate at acid pH values with pyrophosphate.

It is believed that these difficulties can be met in future work, and that the bulk of the evidence is now sufficient to advance the pyruvate oxidase theory of vitamin B_1 action. In subsequent papers, we intend to deal with the question whether the effect is direct or indirect; our present view is that it is direct and that there is no evidence that it is concerned with anaerobic change in our experiments. Rydin [1935] it will remembered could obtain no evidence at all for the view that vitamin B_1 catalysed any anaerobic reaction with methylene blue.

Another interpretation is now given to the experiments of Quastel & Wheatley [1932], with the autoxidized brain tissue; it must be true as they thought that they were studying the oxidation of pyruvate (or lactate), but the extent of this oxidation does not depend only upon the actual oxidation level before adding the substrate; it is also a function of the inherent stability of factors in the tissue as influenced by the preliminary period; the effect of traces of substrate in this period cannot be neglected.

In case there may still be those who are uncomfortable about the applicability of these results upon teased brain to those obtained with organized slices of tissue, we may stress the similar behaviour of pyruvate *in vivo* and *in vitro*, the fact that we can show up calcium effects in the teased tissue, and more important still that the phenomena as regards pyruvate can be demonstrated perfectly well with slices of tissue. There is really no ground at all for thinking that the particular reaction of pyruvate involving the vitamin which has been here studied does not take place *in vivo* or in the more highly organized tissue, or that the results are not applicable. This does not mean that in the more organized tissue slice some of the products of oxidation may not be brought into contact with other enzyme systems, and so modified. It is likely that the results are general for brain, as O'Brien and Peters showed formation and disappearance of pyruvate from lactate in rat's brain; and very recently Sherman & Elvejhem [1936] report a study upon chicken's brain which confirms in detail the work of the author and Thompson [1934].

In connexion with work upon the relation of the action of vitamin B_1 and its chemical structure, it is noteworthy that the action of the vitamin can be exerted at an acid reaction as well as at a *p*H at which there will be a profound alteration of the ionization of the quaternary grouping. Hence a direct relation to the action of this grouping is not indicated, though it is hard to believe that the two are not related. Possibly the ionization at the surface of the enzyme is changed as compared with that in solution; the author has shown elsewhere [1931] that this is so for an amine group at an interface; the apparent dissociation constant was found to be altered by 3 *p*H units for palmitic amide at the interface benzene-buffer solution. If this applies to the quaternary group of the vitamin the region of dissociation would be shifted to the acid side, i.e. in the right direction.

Summary of effects with pyrophosphate.

The following facts must be covered by an explanation of the action of pyrophosphate.

At acid p*H values.* Lactate respiration is initially reduced and later stabilized. Vitamin effects improved. No evidence that pyruvate formation is increased. Pyruvate respiration unaffected. It interacts with lactate.

At p*H 7·3.* Lactate respiration initially reduced, and later improved. Vitamin effect improved later. Pyruvate partly inhibited. Disappearance of α-glycerophosphate improved [Johnson, 1936].

At p*H 7·7.* Lactate respiration inhibited up to 1·5 hours: pyruvate respiration inhibited.

Though the oxidation of lactate via pyruvate is almost a postulated textbook step, it is important to have this clearer proof that it is an important channel of oxidation for lactate in brain. This work does not decide whether it is the only method of oxidation; a problem which awaits quantitative investigation.

Peters *et al.* [1935] found that from the 2nd to the 3rd hour with avitaminous tissue in lactate solution (*p*H 7·3), poisoned with iodoacetate, 0·924 mg. pyruvate was formed and some 400 μl. O_2/g. taken up. The conversion of lactate into pyruvate would need about 115 μl.; the residual respiration for such a period is usually about 250–300 μl. Hence, the extra O_2 uptake is of the right order for conversion of lactate mainly through pyruvate. Much of the maintenance produced by pyrophosphate with lactate must be due to the modifications in the early rate of oxygen uptake.

EXPERIMENTAL.

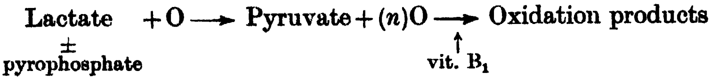
In the experiments here described, the technique of Kinnersley *et al.* [1935, Appendix] has been followed throughout; only essential points are therefore noted in the Tables. Pyruvic acid has been prepared from crystals, kept frozen in cold store; it was made up to be about $5 M$ in amounts of 10 ml. as required and kept at 0°. Samples were neutralized immediately before use, Ringer phosphate being added before the N alkali. Pyrophosphate was also freshly made up, from the dried Na salt. Of late it has been my practice to add the substrate immediately before mashing the tissue, after weighing. After this the tissue can be allowed to stand for some time at room temperature. In several experiments it has been found that the O_2 uptake at 30° and also the vitamin effect are not much influenced, even by a long period of shaking, up to 2 hours in air at 15–20°. Evidently the enzyme systems may be considered to be fixed at this *p*H, so that the only necessity is to allow time for diffusion to take place.

Unless otherwise stated, oxygen uptakes (Barcroft differential technique) are the average of duplicates. Na lactate $0{\cdot}035$–$0{\cdot}04 M$ has been made from Zn lactate; Na pyruvate concentration has been $0{\cdot}020 \pm 0{\cdot}004 M$ according to convenience, and Na pyrophosphate (brought to *p*H used) $0{\cdot}0075 M$. In Exp. 1161 the amounts were: Li lactate $0{\cdot}001 M$, Na pyrophosphate $0{\cdot}01 M$. These variations do not influence the present results. Latterly it has been the practice to neutralize the pyruvic acid with a mixture of KOH and NaOH (N) in ionic concentration equivalent to the rest of the Ringer phosphate to avoid possible K complications [Ashford & Dixon, 1935]. The expression "brain" refers to the mixed cerebral hemisphere and optic lobes. Crystalline vitamin (natural) $2{\cdot}5 \pm 0{\cdot}5 \gamma$ has been added when indicated; the exact amount is not recorded, as the concentration is maximum and wide increase produces no further change.

SUMMARY.

1. Evidence against the conception that lactate is directly oxidized to pyruvate in pigeon's brain tissue as a preparatory stage to complete oxidation has been re-examined and rejected, so that the view can now be advanced that the action of vitamin B_1 is related specifically to pyruvate oxidase in its aerobic reaction.

2. The course of reaction can now be represented as



$$\underset{\text{pyrophosphate}}{\underset{\pm}{\text{Lactate}}} + \text{O} \longrightarrow \text{Pyruvate} + (n)\text{O} \underset{\overset{\uparrow}{\text{vit. B}_1}}{\longrightarrow} \text{Oxidation products}$$

3. The best substrate for demonstrating the catatorulin effect is pyruvate.

4. Lactate and pyruvate oxidase systems can be separated at acid *p*H values.

5. Vitamin B_1 and pyruvate are necessary to ensure stability of pyruvate oxidase at *p*H 6·6.

I am much indebted to R. Wakelin for skilful assistance, to Mr Kinnersley for help, to Dr Westenbrink and Mr Sinclair for criticism and to the Medical Research Council for grants in part aid of these researches.

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CCCX. THE OCCURRENCE OF AN OESTROGENIC SUBSTANCE IN THE SEXUAL SKIN OF MONKEYS.

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Oestrogenic substances can be recovered from the ovaries, placenta, urine, blood, faeces, bile, colostrum, testes, adrenals and pituitary [Doisy, 1932; Callow & Parkes, 1936]. Until recently there has been no evidence of their presence in tissues on which they specifically act. The present paper is an account of their occurrence in the sexual skin of monkeys, a tissue whose activity, it has been found, is controlled by oestrin.

The sexual skin in the female is generally restricted to the circumanal and circumgenital zones. These regions become highly coloured, and in certain species considerably swollen, during the first or follicular phase of the menstrual cycle (for details as to species variation, see Zuckerman [1930]). Male monkeys do not normally exhibit a sexual skin, but it has been found recently that the injection of oestrone induces considerable swelling of the anus, scrotum, and surrounding regions [Dohrn *et al.*, 1933; see Zuckerman & Parkes, 1936, for further references].

Both normal and experimental sexual skin and experimental scrotal swellings appear on section as a jelly-like avascular tissue. The tissue is either colourless or faintly blue; usually it is odourless, but occasionally it smells unpleasantly. The cut surfaces exude a pale straw-coloured grumous fluid, which in amount varies with the extent of surface and the degree of swelling.

The effect which sexual skin swelling has on the water balance is discussed elsewhere [Krohn & Zuckerman, 1936].

Material and technique.

Tissues from three female Hamadryas baboons (*Papio Hamadryas*), a female Chacma baboon (*P. porcarius*), a male Hanuman langur (*Presbytis entellus*), and sixteen male and female rhesus monkeys (*Macaca mulatta*), were used in this investigation.

Sexual skin swelling in baboons is considerable, and is confined to the skin immediately surrounding the vulva and anus. Corresponding changes do not occur in the Hanuman langur. In the rhesus monkey the changes vary with age. In the pubertal female the first phase of sexual skin activity mainly takes the form of a blister-like swelling of the tissues immediately ventral to the pudendal cleft, the swelling being somewhat pear-shaped. The body of the pear is in front, and is formed by the expansion of a transverse pubic flap of skin, usually referred to as the scrotal folds; the apex of the neck of the pear is formed by the praeputium clitoridis. The degree of blister swelling decreases in successive phases of sexual skin activity, and in adolescence the pubic swelling is not so much oedematous as rugose, and the area affected extends more and more away from the genital region. In some cases, lobulated swellings reach well above the iliac crests. In

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> fully mature rhesus females swelling of the sexual skin is unusual, the only obvious change undergone by the skin being one of colour. The extent of the area that colours is variable, but the most intense coloration occurs in the sexual skin proper, the area immediately surrounding the pudendal cleft and anus.
>
> The corresponding changes that can be experimentally induced in the young male rhesus show the same variations in character relative to the length of the time during which oestrin is administered. The first change noticed, towards the end of a week's injections of 100 γ of oestrone daily, is a thickening of the scrotal skin and an eversion of the anal margin. From that time onwards the changes are rapid. By the middle of the second week the anus usually forms a prominent tense and deep-red swelling, which begins to be resorbed about the end of the second week of injections. The scrotum and penile sheath become more and more swollen, the swelling, as in the pubertal female, being pale pink and somewhat translucent. As the period of injections extends, the scrotal swelling decreases, and the reactive area, as in the female, becomes more extensive.

The material examined comprised sexual skins, exudate of the sexual skin, and a number of other tissues, all of which are denoted in Table I.

Table I. *Tissues tested for oestrogenic action.*

Species	Number	Sexual skin or scrotum	Sexual skin exudate	Blood serum	Liver	Kidneys, spleen, pancreas	Striated muscle	Extra-genital skin	Mammary gland
Papio Hamadryas	E.U. 2	×	—	—	—	—	—	—	—
,,	E.U. 4	×	×	—	—	—	—	—	—
,,	E.U. 5	×	—	×	—	—	—	—	—
Papio porcarius	O.M. 105	×	×	×	×	×	×	—	—
Presbytis entellus	O.M. 82	—	—	×	—	—	—	—	—
Macaca mulatta	O.M. 60	×	—	—	—	—	—	—	—
,,	O.M. 63	×	—	×	—	—	—	—	—
,,	O.M. 72	×	—	×	—	—	—	—	—
,,	O.M. 73	×	—	—	—	—	—	—	—
,,	O.M. 24	×	—	—	—	—	—	—	—
,,	O.M. 36	×	—	—	—	—	—	—	—
,,	O.M. 79	×	—	×	×	×	×	×	×
,,	O.M. 19	×	—	—	—	—	—	—	—
,,	O.M. 23	×	—	—	—	—	—	—	—
,,	O.M. 25	×	—	—	—	—	—	—	—
,,	O.M. 40	×	—	—	—	—	—	—	—
,,	O.M. 41	×	—	—	—	—	—	—	—
,,	O.M. 42	×	—	—	—	—	—	—	—
,,	O.M. 135	×	—	—	—	—	—	—	—
,,	O.M. 136	×	—	—	—	—	—	—	—
,,	O.M. 137	×	—	—	—	—	—	—	—

Tissues from both normal and experimental animals were used, some of the normal animals being in an active, others in a quiescent sexual skin phase. The experimental animals had been injected either with oestrone alone, with both oestrone and progesterone, with oestrone and cholesterol, or oestrone and epicholestanol (the injections having been given primarily for the purpose of another investigation). Except where otherwise stated in Column 4 of Table II, all injections were given daily for 14 days.

Animals which had been injected with two preparations were included in the present investigation since the results of the injections showed plainly that the effects of oestrone dominated those of the other injected substance—at least in so far as external changes are concerned. The character of the scrotal and anal swellings in these animals was no different from that in animals injected with oestrone alone.

Care was taken in the treatment of the experimental animals to keep the point of injection as far from the scrotal region as possible, so as to obviate any

Table II. *Condition of animals studied.*

Designation of animal		Body wt. (kg.)	Oestrone (γ/day)	Progesterone (γ/day)	Epichotestanol (mg./ day)	Cholesterol (mg./ (day	Days of treatment	Degree of sexual skin swelling at death	Oestrogenic activity of extracts of sexual skin
O.M. 24	♂	3·4	50	—	—	—	14	Moderate	Negative
O.M. 36	♂	2·5	50	—	—	—	14	Pronounced	,,
O.M. 79	♂	2·9	100	—	—	—	15	,,	Positive
O.M. 19	♂	3·1	200	500	—	—	14	,,	Negative
O.M. 23	♂	3·1	50	400	—	—	14	Slight	Positive
O.M. 25	♂	4·3	50	300	—	—	14	Pronounced	Negative
O.M. 40	♂	4·1	50	500	—	—	14	,,	,,
O.M. 42	♂	4·0	50	1000	—	—	14	,,	,,
O.M. 135	♂	2·1	100	—	—	5	14	Moderate	,,
O.M. 136	♂	2·4	100	—	5	—	14	Pronounced	,,
O.M. 137	♂	3·0	100	—	10	—	14	,,	,,
E.U. 4	♀	10·0	Normal	—	—	—	Not treated	Full	Positive
E.U. 2	♀	10·7		—	—	—		,,	Negative
O.M. 105	♀	14·6		—	—	—		Abnormally persistent	Positive: slight
E.U. 5	♀	—		—	—	—		None	Negative
O.M. 60	♀	4·9		—	—	—		,,	,,
O.M. 63	♀	3·4		—	—	—		,,	,,
O.M. 72	♀	3·4		—	—	—		,,	,,
O.M. 73	♀	5·4		—	—	—		,,	,,
O.M. 82	♂	4·1	200	—	—	—	39	,,	—
O.M. 41	♂	3·6	50	750	—	—	14	,,	Negative

possibility of the introduction of the hormone into tissue which was later to be extracted. Injections were given intramuscularly.

The cutaneous and subcutaneous tissues comprising the sexual skin or scrotum were completely dissected at autopsy from the underlying muscle. The swollen sexual skins in the three baboons, E.U. 2, E.U. 4 and O.M. 105, weighed 0·9 kg., 1·092 kg. and 3·574 kg. In the case of the male rhesus monkeys the anus and surrounding skin when swollen were occasionally included with the scrotum, the maximum weight of oedematous tissue extracted in the case of any male monkey being 165 g.

With the exception of two samples of exudate from the sexual skin, and certain of the blood sera, which were injected into test mice without previous extraction, all the tissues tested were first extracted for oestrin according to the method outlined by Parkes & Bellerby [1926].

EXPERIMENTAL RESULTS.

The preparations were tested for oestrogenic activity by the standard method on gonadectomized mice. Control tests were also run, with the same technique of injection and vaginal examination, using a suitably diluted solution of crystalline oestrone, kindly provided by the Department of Biological Standards, National Institute for Medical Research.

Clear positive results were obtained only with the sexual skin (or scrotum) of O.M. 23, E.U. 4 and O.M. 79, with the exudate of E.U. 4, and the livers of O.M. 79 and O.M. 105.

Sufficient material for complete biological assay was available only in the case of the first two, which contained the equivalents of 95 γ and 27 γ per kg. of tissue respectively. The amounts present in the other four were in the region of 6 γ per kg., 67 γ per litre of exudate, and 15 γ and 7 γ per kg. respectively.

Evidence of some oestrogenic activity (i.e. pro-oestrous smears) was also provided by several of the other tissues examined (e.g. O.M. 105, sexual skin, O.M. 105, exudate of sexual skin). Completely negative results were obtained with the blood serum of O.M. 82, using amounts of extract equivalent to 11 ml. of serum, and with the blood serum of O.M. 79 (7 ml. of serum). A pro-oestrous smear was obtained with the equivalent of 50 ml. of the blood serum of O.M. 105. The equivalent of 60 g. of the striated muscle of O.M. 105 proved negative, as was also the equivalent of 30 g. of the extra-genital skin of O.M. 79. No indications of oestrogenic potency were given by extracts of the tissues of any of the normal animals which at autopsy were in a quiescent sexual skin phase.

Our failure to obtain more conclusive results was due not only to the small amounts of tissue available for extraction but also to the difficulty of securing emulsions for injection which were on the one hand sufficiently concentrated and on the other sufficiently fluid.

Discussion.

Since our methods of extraction were relatively crude and did not include preliminary hydrolysis, the many negative results we obtained cannot be taken to indicate that the tissues concerned were free of oestrogenic substance. On the other hand, our positive results show that some free oestrin, the term being used to denote unspecified oestrogen, is present in the sexual skin, the fluid of the sexual skin and the liver. Since several swollen sexual skins gave negative results, no particular weight can be attached to the fact that negative results were always obtained with tissues from animals whose sexual skins were inactive. Moreover our data do not provide any explanation for the varying amounts of oestrogen found in the swollen sexual skins or oedematous scrota we examined. It is significant, however, that positive results were obtained with both the naturally and the experimentally active circumgenital skin, and that four out of fourteen swollen skins yielded active extracts, whereas none of the seven which were not swollen gave positive results.

The completely negative result obtained with the blood serum of O.M. 82 should be noticed in the evaluation of these facts. Frank *et al.* [1932] have shown that intravenously injected oestrin very rapidly ceases to be identifiable in the blood. Fee *et al.* [1929] found the same with the help of a heart-lung-kidney preparation. If no oestrogen could be extracted from the blood of an animal which had received as much oestrone as O.M. 82 had (200γ daily for 39 days), it is very unlikely that the oestrogenic substance we did succeed in extracting from three sexual skins and two livers was in solution in the blood contained in these organs.

It is well known that large amounts of oestrin are present in the urine of pregnant women. Cohen *et al.* [1935] found that only 1 % of the oestrin present during the first 8 months of gestation is "free", the rest being "combined" (in the case of oestriol, in the form of an oestriolglucuronide; Cohen & Marrian [1936]). According to Zondek [1934], most of the oestrin injected into an experimental animal is converted into the "combined" form, and then stored in striated muscle. He believes that the process of combination takes place in the liver, and our finding of "free" oestrin in that organ may be related to his view. Frank *et al.* [1935] have recently been able to liberate, by some method of which the steps are not given, large amounts of oestrogen from striated muscle (up to 8000 M.U. per kg. dried muscle). A similar amount was extracted from a human uterus removed during the intermenstrual period; and, by postponing ether extraction to the later stages of purification, the yield from blood has also been increased fourfold [Frank & Goldberger, 1935]. In the circumstances it is reasonable to suppose that the yield from an active sexual skin would be increased after preliminary hydrolysis; we propose to investigate this point as soon as further material and facilities become available.

The possibility that the presence of "free" oestrin in the sexual skin and in its interstitial fluid is indicative of selective concentration at a site of action requires investigation. It is not unlikely that oestrin is "absorbed" by the cellular elements of the swelling in the sense in which Zondek states it is by striated muscle. If it were, it would presumably be absorbed in the "combined" form. Accordingly, if the sexual skin proves to be a localized part of the body's subcutaneous tissues in which oestrin is stored, it may prove to be possible to relate the oedematous process which it undergoes to the fairly generalized oedema which is a not uncommon occurrence in human pregnancy, during which the major part of the oestrin elaborated becomes combined, presumably in the tissues.

Our finding of "free" oestrin in a tissue which is specifically sensitive to the hormone is also of interest in view of the opinion that the occurrence of oestrogenic substances in benign tumours is indicative of their aetiological responsibility for the condition [Geschickter *et al.*, 1934; Ingleby, 1935; Witherspoon, 1935]. The practical importance of this view clearly indicates the necessity for the further investigation of the probable normal selective concentration of oestrin at a site of action.

SUMMARY.

Various tissues of eight normal monkeys and thirteen monkeys which had been injected with oestrone were tested for oestrogenic potency. Positive results were obtained with the active sexual skin, the exudate of the sexual skin, and the liver.

The monkeys and baboons used in this study were bought with the aid of grants to S. Z. from the Medical Research Council and the Eugenics Society respectively. We wish to express our best thanks to Dr A. E. Russell for her help with the earlier tests and to Mr A. E. Wilhelmi for his assistance with the extractions.

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CCCXI. THE RESORPTION OF THE BILE ACIDS FROM THE INTESTINES.

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The circulation of the bile acids has been recognized for some time. Schiff [1870] showed that bile given *per os* acted as a cholagogue. Tschernoff [1884] pointed out that the cholic acids were partly resorbed from the intestines, carried to the liver and again secreted with the bile. Later this inference was made and experimentally supported by several investigators. Copeman & Winston [1889] and Pfaff and Balch [1897] showed that this circulation probably occurs even in man. From experiments with bile-fistula dogs Whipple & Smith [1928] concluded that of the bile acids normally excreted only about one-seventh was freshly formed, the rest having been resorbed and then carried to the liver.

Very little is known, however, about the mechanism of this resorption. Verzar & Kuthy [1930] have shown that the bile acids from the intestinal contents are partly adsorbed to the surface of the mucosa, whence they pass the outer cellular walls together with the fatty acids as water-soluble or molecular-disperse choleic acids. These compounds, however, must be at least partly decomposed already in the epithelial cells, because here fat globules in great quantities are microscopically visible after a fat meal. The liberated bile acids then, according to Verzar [1931], return to the lumen surface of the cells to combine with new fatty acids. Thus this theory accounts only for the first step in the resorption of the bile acids themselves.

The purpose of our experiments was to try to trace the further paths followed by the resorbed bile acids from the intestinal mucosa to the liver; either they might follow the fats in the lymph through the thoracic duct in the form of free bile salts or as choleic acids, or they might diffuse through the walls of the blood capillaries into the portal system. In the former case they would have to be mixed with the systemic blood before reaching the liver; in the latter case they would be carried by the portal blood directly to the liver. The scanty data in the literature bearing on this question give very conflicting information.

EXPERIMENTAL.

In our experiments we have, after laparotomy, injected strong bile acid solutions into the small intestines of animals. By following the bile acid contents of portal blood as well as of systemic (heart) blood we believe that we have been able to trace the paths of these acids.

The animals were anaesthetized during the whole experiment. The samples of systemic blood were always taken by heart puncture (with the exception of the horse, Table III). The portal samples were taken after laparotomy with a syringe with a very fine curved needle, and the hole was instantaneously closed with a small clamp. Before the injection we always took initial samples both of systemic and portal blood. The bile salts used were prepared according to O. Hammarsten and were very pure; some sodium phosphate was always added

Table I.

Date	Animal	Wt. kg.	Anaesthetic	Injected into the intestines	Cholic acids in blood									
					Initial value		After injection							
							1 hour		2 hours		3 hours		4 hours	
					h.	p.	h.	p.	h.	p.	h.	p.	h.	p.
8. x.	Rabbit 2	1·8	Pernocton	10 ml. Na cholate 5%	1·0	—	2·2	6·3	2·4	3·6	—	—	—	—
13. v.	Rabbit 3	2·9	Pernocton	10 ml. Na cholate 5%	1·0	3·0	4·4	14·2	3·8	14·2	—	—	—	—
4. vi.	Rabbit 15	3·6	Ether	10 ml. Na cholate 5%	1·1	3·9	1·9	6·6	2·1	6·6	—	—	—	—
4. vi	Cat 4	3·3	Avertin + ether	10 ml. Na cholate 5%	2·1	5·3	7·9	13·3	3·3	8·5	2·8	7·7	—	—
15. vi.	Rabbit 28	2·7	Ether	10 ml. Na glycocholate 10% + Na stearate 5%, + glycerol 5%	1·3	2·8	1·4	8·6	1·7	5·1	—	—	—	—
17. vi.	Cat 9	3·7	Urethane	10 ml. Na glycocholate 10% + Na oleate 5% + glycerol 5%	1·2	3·1	1·7	5·0	1·5	5·5	2·0	3·0	1·9	5·1
19. vi.	Cat 10	3·2	Urethane	10 ml. Na glycocholate 10% + Na stearate 5% + glycerol 5%	1·1	2·1	1·1	4·3	1·4	6·0	2·5	5·0	1·1	4·8

Table II.

Anaesthetics in both cases: avertin + ether.

Animal	Date of ligation	Date of exp.	Wt. kg.	Injected into the intestines	Cholic acids in blood									
					Initial value		After injection							
							1 hour		2 hours		3 hours		5 hours	
					h.	p.	h.	p.	h.	p.	h.	p.	h.	p.
Cat 6	19. vi.	28. vi.	3·3	(1) 10 ml. water	5·2	5·0	5·0	5·2	—	—	—	—	—	—
				(2) 10 ml. Na cholate 5%	5·0	5·2	10·8	19·8	24·0	—	22·3	—	—	—
Cat 7	26. vi.	9. vii.	3·5	10 ml. Na cholate 5%	16·5	15·7	23·7	31·0	25·2	39·0	22·0	24·4	13·9	17·6

to the solutions. The solution was injected through a fine needle into the jejunum. Between the portal blood-samplings the laparotomy was closed with clips so that the intestines should not become cold or dry. All the experiments were made between 12 noon and 6 p.m. The bile acid analyses were carried out according to Josephson [1935].

All the figures for the bile acid content of the blood are expressed as mg. per 100 ml. In the tables h. signifies systemic (heart) blood and p. means blood from the portal vein.

In Table I are given the results of some of the experiments on healthy animals.

The cholic acid values in the portal blood were always significantly increased after the injection of the bile salt solution into the intestine. In most cases the values in the systemic blood were also slightly increased. It must be mentioned that Greene *et al.* [1928] also found an increase of the bile acids in the portal blood after administration *per os*; their methods however were not very reliable.

In the hope of obtaining more decisive figures we have also tried to inject bile salt solutions into the intestines of animals with jaundice, caused by ligation of the common bile duct some time before the experiment. This plan was unsuccessful with rabbits, since they usually became very weak when jaundiced and always died during the second operation. With cats we had more success, and in Table II are given the results with two of these animals; cat 6 first received an injection of water as a control.

The increase of the bile acid content of the blood is of course much larger in these experiments in which their elimination was delayed, but the difference between portal and heart bloods is of the same order of magnitude as in the previous table.

As a complement to the blood values after resorption, we also wanted to analyse the chyle after injection of cholic acid into the intestines. For this purpose we could not use small animals, since 5 ml. of lymph were necessary for an analysis. We therefore examined samples of thoracic lymph from five different recently slaughtered cows. To our surprise we found that all the samples were devoid of detectable quantities of cholic acids, all the values being within the limits of error of the method, that is between 0 and about 0·3 mg./100 ml., yet cows normally have a rather high bile acid content in the blood, usually 3–4 mg./100 ml. It may be mentioned that with the method employed even those cholic acids are determined which are conjugated as choleic acids.

For the purpose of getting a lymph analysis after injection of bile salts into the intestines we now used a horse as test animal. The horse has a very large cisterna chyli, and the thoracic duct has a diameter of about 5 mm. We thus succeeded in getting 35 ml. lymph, 80 min. after the bile acids had been injected and shortly after the killing of the animal.[1] As anaesthetics chloral and

Table III.

17.i. Horse, about 500 kg. chloral + chloroform.

Injected into the intestine	Blood				Lymph	
	Initial value		1 hour			
	h.	p.	h.	p.	Chol. acids mg./100 ml.	Lipoids %
2 l. Na glycocholate 10% + oleic acid 5%	1·3	2·3	2·2	6·4	0, 0·2, 0, 0·4	1·65, 1·78
				Average	0·15	1·71

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[1] We are indebted to Prof. Forssell, Prof. Palmgren and Dr Dolk at the Veterinary High-school of Stockholm for their kindness in helping us to carry out this experiment.

chloroform were used. The lipoid content of the lymph was determined by extraction with alcohol-ether (3:1), evaporating and weighing. The results are shown in Table III.

Even the horse lymph proved to be absolutely free from cholic acids after the injection into the intestine, in spite of the fact that the lipoid value was fairly high, and though the bile acids were evidently increased in the portal blood.

DISCUSSION.

In a paper which will soon appear we propose, together with Jungner, to report experiments which show that even large amounts of bile acids injected into a peripheral vein of a healthy animal disappear from the blood almost immediately. If the acids are injected into a vein of the portal system the content even in heart blood is markedly higher.

For this reason we may suppose that if the bile acids were resorbed only by the lymph vessels and carried to the peripheral blood, we would scarcely have found any rise at all in the blood content. If they were resorbed by the portal vein, however, we might have found an increase in the portal and possibly also in the peripheral blood. The latter is indeed the case, as Table I shows, and the increase corresponds to a much higher resorption than would appear from a superficial estimate. Schwiegk [1932] has shown that the velocity of flow in the portal vein can be increased more than 200 %, for instance by cooling or by the presence of (especially fat) food in the intestines. Even a rise in the blood bile acids increases this velocity, which of course must again decrease the cholic acid content.

This does not exclude the possibility that there is a simultaneous resorption by the lymph vessels. If however a bile salt solution is injected peripherally into an animal with the bile duct ligatured, there will appear a much larger and more prolonged increase of the blood bile acid content than in normal animals. The figures in Table II show that even in animals thus jaundiced the elevation of the bile acid content of the systemic blood is evidently lower than that of the portal blood after resorption of bile acids.

The final evidence that cholic acids are not resorbed by the lymph vessels is given by the analysis of the lymph samples from cows and a horse, where no cholic acid at all could be found, even after injection into the intestines of 200 g. Na glycocholate and during resorption of fats.

By the experiments related above we believe it to be demonstrated that the bile acids, when being resorbed, do not follow the fatty acids through the lymph vessels but diffuse into the portal blood and are carried to the liver. This means that the choleic acids must be decomposed into fatty and bile acids immediately after passing the epithelial layer of the mucosa, and that the two substances thus after the common resorption follow different paths.

This may also have a clinical interest for bile acid therapy which is now frequently used. When the bile acids are given *per os* they will probably never appear in the peripheral blood if the liver is undamaged, whilst in certain cases of liver injury they may possibly be found there in a low concentration.

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CCCXII. SEPARATION OF SERUM ALBUMIN INTO TWO FRACTIONS. I.

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The differentiation of proteins is made uncertain by their complexity and instability, by the difficulties of separation and by the inapplicability of many of the usual criteria of chemical purity. For example, the position with regard to the existence and interrelation of different serum globulins is still highly controversial [Hardy & Mellanby, 1905; Chick, 1914; Sørensen, 1925; Hewitt, 1927; Mutzenbecher & Svedberg, 1933]. It is generally agreed that serum albumin and globulin are separate and distinct fractions and, since serum albumin can be obtained in a crystalline condition, there should be less difficulty than with most proteins of isolating it in a reasonably pure state. Grave doubts have, however, been expressed from time to time as to whether serum albumin is a single chemical compound or a mixture. Differences in heat coagulability [Halliburton, 1884], solubility [Sørensen, 1930], and carbohydrate content [Hewitt, 1934] have been observed, but despite the indications given in experiments of this kind, definite and conclusive proofs of the existence of different serum albumins have not been easy to obtain. The starting point of the present investigation was the observation recorded in a previous communication [Hewitt, 1934] that by fractionation with ammonium sulphate it was possible to separate albumin into fractions of widely different carbohydrate content. The present communication describes the separation of horse serum albumin into two fractions which can be distinguished readily by chemical or physical methods.

Methods of fractionation.

The aim of the experiments was to separate albumin into two main fractions, the crystalline fraction which separates first from ammonium sulphate solutions and the most soluble fraction remaining in the mother-liquors.

The general method of obtaining crystalline albumin has not been altered fundamentally for over 30 years [Hopkins & Pinkus, 1898]. Frequent alterations in *p*H tend to have deleterious effects on serum proteins, so the procedure of Adair & Robinson [1930] of dissolving the crystals in ammonium acetate solution and reprecipitating with acid was avoided. Prolonged dialysis is a further source of possible change in the proteins and this was also obviated. Although alcohol-ether extraction at low temperature is an efficient method of obtaining stable preparations of proteins in the dry state [Hardy & Gardiner, 1910; Hartley, 1925; Hewitt, 1927], this procedure was rejected on the ground of possible criticism [McFarlane, 1935].

The main points in the present method are that oxalate plasma was used instead of serum, that the plasma was diluted with an equal volume of water before addition of saturated ammonium sulphate solution, that the globulins were removed as usual by 50 % saturation with ammonium sulphate, and that crystallization in the 50 % saturated ammonium sulphate filtrate was effected by addition of acetic acid and not sulphuric acid.

In order to save space details will be given of only one fractionation process, in which fractions A_3, B, C and D were isolated.

The plasma from oxalated horse blood was diluted with an equal volume of water, saturated ammonium sulphate solution was added to make the mixture 50 % saturated with ammonium sulphate and the globulins were removed by filtration. To 100 l. of the clear filtrate (corresponding to 25 l. of plasma) were added 160 ml. of glacial acetic acid. After a short time crystallization commenced and this was allowed to continue overnight. The crystals were filtered off and redissolved in 10 l. of water and insoluble green-coloured material was filtered off, 9·5 l. of clear filtrate being obtained. To this was added saturated ammonium sulphate solution (5·7 l.). The crystals which separated were filtered off and redissolved in 3 l. of water, the solution being clarified by passing it through a Sharples centrifuge. 1650 ml. of saturated ammonium sulphate solution were added to the clear solution and the fine needles which separated out were filtered off, dried in towels and dialysed in cellophane bags against running water for 48 hours. In this way fraction A_3 (yield about 73 g., calculated as dry weight of protein) was obtained.

To the filtrate of the first crystallization (97 l.) was added more saturated ammonium sulphate solution (20 l.) and glacial acetic acid (100 ml.). The precipitate produced (fraction B) was dried and dialysed as described in the case of fraction A_3, the yield being about 76 g. To the filtrate of the second crystallization 5 l. of saturated ammonium sulphate solution and 50 ml. of glacial acetic acid were added and the precipitate was collected as described previously. In this way fraction C, yield about 73 g., was obtained. The filtrate from the third crystallization (4 l.) was also treated with saturated ammonium sulphate solution (2·5 l.) and fraction D was obtained (yield 4·8 g.).

As a result of other fractionations the following fractions were obtained: E_8 was a crystalline albumin obtained from 100 l. of the 50 % saturated ammonium sulphate filtrate (corresponding to 25 l. of horse plasma). The fraction had been recrystallized eight successive times and was obtained in a yield of about 117 g. One quarter of this preparation was subjected to four further recrystallizations making twelve recrystallizations in all, this yielding fraction E_{12}.

Fraction H_1 (50 g.) was a soluble fraction obtained from the mother-liquors of 100 l. of 50 % saturated ammonium sulphate filtrate after two less soluble fractions F_4 and G had been removed. F_4 appeared to contain globulins and was not further investigated, whilst the intermediate fraction G was obtained in a final yield of about 147 g.

Nitrogen content and protein determinations.

The discrepancies between the figures given for the nitrogen content of proteins by different investigators are very remarkable. Two comparatively recent investigations in which this matter received detailed consideration yielded very different results. For serum albumin Adair & Robinson [1930] found a nitrogen content of 15·6 %, whilst Smith *et al.* [1932] give a figure of only 13·8 % of nitrogen. It appears that there must be some difference in experimental conditions to account for the divergence.

For nitrogen determinations in the present investigation a modification of the micro-Kjeldahl method using the Parnas and Wagner apparatus was employed. The dry weights of the proteins were determined in some cases by drying *in vacuo* over sulphuric acid and in others by coagulating the protein by heating at the isoelectric point and drying with alcohol and ether. The nitrogen content observed in the case of crystalline fractions was from 14·1 to 14·4 % (mean 14·2 %). In the case of the very soluble fractions however the nitrogen content is much lower (about 12·9 %). The low nitrogen content of the soluble fractions is explained, as will be shown later, by the very high carbohydrate content of these fractions but it is surprising that the crystalline fractions have so much lower nitrogen contents than those of Adair and Robinson. Since the nitrogen content has been an incidental determination in the present investigation, whilst Adair and Robinson directed special attention to the point, it is not advisable unduly to stress the present figures. The specific refractive increment was determined roughly with an Abbé refractometer and gave a mean value of 0·00177. This value is lower than Adair & Robinson's figure of 0·00183 but again these investigators devoted much greater attention to the point.

Carbohydrate contents of fractions.

The carbohydrate content was found to be a valuable index to the course of the fractionation. As described previously [Hewitt, 1934] the method used for determining carbohydrates was that of Sørensen & Haugaard [1933]. It may be reiterated here that the solutions to be used for photometric examination must be kept shielded from light and that the values for the protein blank determinations are unreliable and may be ignored. As the result of the combined work of the investigators just mentioned and of Fränkel & Jellinek [1927], Levene & Mori [1929], Rimington [1931] and Bierry [1934], it seems probable that the carbohydrate group present in serum proteins is a polysaccharide containing equimolecular amounts of galactose, mannose and glucosamine. No results obtained during the present work presented any serious evidence to contradict the conclusions reached by other workers. Hence all results quoted will be based on the assumption that the carbohydrate present is galactose-mannose-glucosamine (for brevity referred to as g.-m.-g.). The protein content was calculated with the use of the protein: nitrogen factor 7·0. The carbohydrate content of the total albumin fraction of serum is usually about 2·8 % (g. of g.-m.-g. per 100 g. of protein). The fractions now described have the following carbohydrate contents:

	E_{12}	E_8	A_3	G	C	D	B	H
Carbohydrate	0·05	0·08	1·18	1·80	2·46	3·96	7·3	8·5 %

The fractions may be divided roughly into three classes: (i) the highly purified crystalline fractions E_{12} and E_8 with so small a carbohydrate content that it cannot be determined accurately, and may be ignored; (ii) the intermediate fractions; and (iii) the soluble fractions B and H from the mother-liquors containing up to 8·5 % of carbohydrate. Our chief attention will be devoted to the two end fractions since the intermediate fractions behave as mixtures of the high- and low-carbohydrate content fractions. In each case the crystalline, sparingly soluble fractions have the low carbohydrate content (in the most highly purified fractions no appreciable amount) and the soluble fractions from the mother-liquors have high carbohydrate contents. Fraction B was diluted, and heated in a boiling water-bath at the isoelectric point to coagulate the protein and the precipitate was centrifuged down and washed with alcohol and ether. When redissolved the coagulum was found to contain 7·8 g. of g.-m.-g. per 100 g. of protein, so that the polysaccharide had not been removed by boiling water, alcohol or ether. When hydrolysed with boiling hydrochloric acid for a few minutes no glucosamine could be detected in E_8 using the method of Elson & Morgan [1933]. With B, glucosamine was detected but could not be determined accurately owing to the large amount of humin formation during the hydrolysis.

Humin formation.

It has been suggested [Gortner, 1916] that humin formation is due to the interaction of carbohydrates and certain amino-compounds. It is not surprising therefore that E_8 and E_{12}, which are free from carbohydrates, remained colourless when hydrolysed with hydrochloric acid whilst B and H, with their high carbohydrate content, gave rise to large quantities of humin under the same conditions.

Optical rotations.

The value of optical rotatory powers in differentiating optically active compounds suggested its use in the investigation of the albumin fractions. A Bellingham and Stanley polarimeter fitted with direct-vision spectroscopic eyepiece was used, and a mercury vapour lamp provided the light source. The mercury green line ($\lambda = 5461$ Å.) was used throughout the measurements.

It will be seen in Table I that the rotations measured varied from $[\alpha]_{5461}$ $-57{\cdot}1°$ in the case of H up to $-70{\cdot}8°$ for E_{12}. The most soluble proteins had the lowest rotatory powers and the crystalline specimens the highest rotations, there being a graded variation amongst the intermediate fractions.

Table I. *Some properties of the different fractions.*

Fraction	Carbohydrate content. g. of g.-m.-g. per 100 g. protein	Optical rotation. $[\alpha]_{5461}$	Van Slyke amino-nitrogen. g. per 100 g. protein
E_{12}	0·05	−70·8°	1·07
E_8	0·08	−70·2	0·90
A_3	1·18	−66·5	0·87
G	1·80	−66·8	0·85
C	2·46	−61·0	0·82
D	3·96	−60·3	0·79
B	7·3	−57·2	0·66
H	8·5	−57·1	0·65

Two observations on these figures should be made here. First, the proteins were not treated with alcohol and ether as described in a previous communication [Hewitt, 1927] for reasons stated in a previous section and hence the optical rotations are not directly comparable with those in the former paper. Second, the calculation of specific rotatory power is based on the nitrogen content of the proteins, using a constant protein: nitrogen factor of 7·0. If the dry weight of the proteins be used the difference between the crystalline albumin and the soluble fractions is even greater, since the specific rotation of fraction H, for example, is reduced to $-50{\cdot}1°$ whilst the figures for the purest crystalline specimens remain at $-70{\cdot}8°$.

Titration curves.

Titration curves indicated a higher base binding capacity by the purest crystalline fractions than by the more soluble mother-liquor fractions. This difference was greater when formaldehyde was added before the titration. Typical curves for the fractions E_8 and H in the presence of about 7 % formaldehyde are given in Fig. 1. The glass electrode was used for these measurements.

Van Slyke amino-nitrogen.

Since the electrometric formaldehyde titration curves indicated a difference between the different fractions, amino-nitrogen determinations were performed using the Van Slyke micro-method. The nitrous acid was allowed to react for 4 min. at 20°. It will be seen in Table I that there is a gradation in the free amino-nitrogen figures of the different fractions, the purest crystalline albumins having the highest amino-nitrogen content ($E_{12} = 1{\cdot}07\ \%$ amino-nitrogen) whilst the most soluble fractions had the lowest figures ($H = 0{\cdot}65\ \%$). The results are quoted in terms of g. of amino-nitrogen per 100 g. of protein.

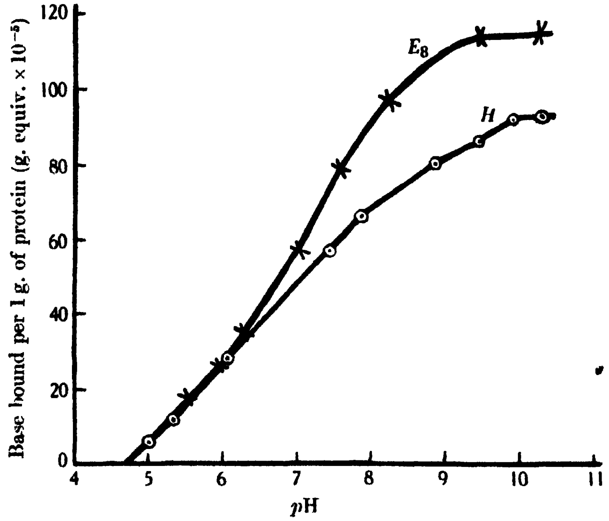

Fig. 1. Titration curves of fractions in presence of formaldehyde.

Heat coagulation.

Protein solutions were diluted to contain about 1 % of protein in 0·85 % sodium chloride solution, the *p*H was adjusted to a value of 4·7 to 4·8 and the solutions were slowly warmed in a water-bath. The crystalline albumin fractions began to coagulate at 60° but the most soluble fractions B and H behaved very differently. It was particularly difficult to coagulate H; very careful adjustment to the isoelectric point had to be made. Some coagulation began in these fractions at about 80° but the supernatant fluid was opalescent and not clear, and the coagulum was not granular but of soft consistency.

The fact that the crystalline low-carbohydrate fractions coagulate at 60° whilst the soluble high-carbohydrate fractions did not coagulate below 80° suggested the interesting possibility that separation of the fractions might be effected by coagulating a mixture at a temperature a little above 60°.

Hence a sample of albumin containing the total albumin fraction of horse serum (carbohydrate content 2·6 g. of g.-m.-g. per 100 g. of albumin) was subjected to a fractional coagulation process. The first coagulation was effected by heating at 62–64°, the coagulum I was centrifuged down, the mother-liquor was reheated and coagulum II collected by heating at 64–66° and so on. The precipitates obtained were dissolved in dilute alkali and analysed for nitrogen and carbohydrate content. The results are given in Table II.

Table II. *Fractional coagulation of sample of serum albumin.*

Fraction	Coagulation temperature	Volume of solution ml.	Nitrogen content %	Carbohydrate g. of g.-m.-g. per 100 g. protein
I	61–62°	5	0·196	3·8
II	64–66°	2	0·038	4·8
III	72–74°	2	0·030	2·1
IV	77–81°	2	0·016	2·7
V	93–100°	2·2	0·008	4·5
M. L.	Not coagulated	12	0·006	8·2

The results of the experiment were not those that would be expected from the low coagulation temperature of the low-carbohydrate content albumin. Evidently even the first coagulum at the lowest temperature carries down appreciable amounts of the high-carbohydrate fraction.

When fraction H was partially coagulated by heating at 80° there was a fairly even distribution of carbohydrate between the precipitate and the supernatant fluid. The precipitated protein contained 9·14 % of carbohydrate and the protein in the supernatant fluid contained 7·74 % of carbohydrate (calculated as g. of g.-m.-g. per 100 g. of protein).

It appears not to be possible, therefore, to utilize the differing coagulation temperatures of the separated fractions to effect a separation of the two.

Tryptophan and tyrosine determinations.

The Folin & Marenzi [1929] method was used for determining tryptophan and tyrosine, the only departures from the published method being that in place of one sample of 20 ml. of the hydrolysate, duplicate samples of 5 ml. were used, the final volume for colorimetric comparison being 25 ml. in place of 100 ml., and the colorimetric comparisons were made in a Stufenphotometer instead of a colorimeter. Duplicate analyses agreed extremely well and the photometer was most useful when the hydrolysates were coloured. With care the method yields very consistent results. In a previous paper [Hewitt, 1934] the tryptophan content of serum albumin was found to be from 0·44 to 0·52 %. Folin & Marenzi's figures were 0·52 and 0·53 % of tryptophan in serum albumin. In the literature the figures are generally higher than these.

It is now found that the tryptophan contents of the purest fractions (E_{12} and E_8) of crystalline albumin are 0·26 and 0·30 % respectively. These extremely low values were readily explained when it was found that the most soluble fractions (B and H) from the mother-liquors of the albumin had tryptophan contents of over three times as much, namely 1·0 %. The tryptophan content of a specimen of serum albumin evidently depends upon the proportions present in it of the different albumin fractions.

The tyrosine contents of the different fractions do not vary so much. The purest crystalline specimens had tyrosine contents of 4·74 and 4·79 % respectively compared with Folin & Marenzi's figures of 4·66 and 4·67 % and the author's previous figure of 4·7 %. The soluble fractions H and B had tyrosine contents of 5·38 and 6·06 % respectively.

DISCUSSION.

Before the theoretical implications of some of the conclusions reached are discussed it will perhaps be well to state clearly in outline what results have been obtained. In Table III are summarized the contrasting properties of the two

Table III. *Contrasting properties of two horse serum albumin fractions.*

Description	Crystalline serum albumin	Mother-liquor fraction
Solubility in ammonium sulphate solution	Least soluble fraction	Most soluble fraction
Preparation	Twelve times crystallized	Remaining in mother-liquors after removal of two less soluble fractions
Carbohydrate content (as g.-m.-g.)	0·05 %	8·5 %
Nitrogen content	14·4 %	13·0 %
Van Slyke amino-nitrogen content	1·0 %	0·65 %
Rotatory power $[\alpha]_{5461}$	-70·8°	-57·1°
Coagulation temperature	60°	80°
Tryptophan content	0·26 %	1·0 %
Tyrosine content	4·79 %	5·38 %
Hydrolysis with hydrochloric acid	Remained colourless	Much humin formation

"end" fractions separated from serum albumin, the most sparingly soluble fraction and the most easily soluble fraction being selected. In this connexion the solubility referred to is in ammonium sulphate solution and not in water.

It is clearly proved that horse serum albumin may, by simple methods not involving any rigorous treatment, be separated into two fractions of quite different chemical and physical properties. The crystalline fraction free from carbohydrate is not difficult to identify; it is probably true serum albumin in a higher state of purity than is generally encountered. It seems that serum albumin is usually contaminated with varying amounts of some other protein which is entirely removed only after a quite extensive crystallization process. The outstanding characteristics of "pure" serum albumin apparently are: (1) its solutions are almost colourless, (2) it contains no carbohydrate, (3) humin formation does not occur during acid hydrolysis and (4) it has a low tryptophan content, namely between 0·26 and 0·30 %. This last point is of some interest, for if it be assumed that serum albumin has a molecular weight of 69,000 [Mutzenbecher & Svedberg, 1933], then the presence of one molecule of tryptophan in each albumin molecule would require a tryptophan content of

$$\frac{204}{69,000} \times 100 = 0{\cdot}298\,\%,$$

a figure in remarkable agreement with the experimental determination.

The identity of the second protein is the next point of interest. It is not suggested that the easily soluble fraction B or H represents a pure protein but it seems probable that they are each a mixture of albumin with another protein It is hoped in the future to obtain further evidence as to the nature of this other protein material, which may be serum mucoid. Serum mucoid has not been studied extensively but its marked characteristics appear to be that it is not coagulated on heating, that it has a high carbohydrate content of about 25 %, and that it has a low nitrogen content, 11·9 % [Zanetti, 1897; Bywaters, 1909; Rimington, 1931]. The soluble fraction from serum albumin has a fairly high carbohydrate content (8·5 %) and is not easily coagulated on heating. Apparently, however, when large quantities of serum albumin are present the coagulum of serum albumin produced on heating carries down with it a considerable portion of this almost non-coagulable protein. It is perhaps not surprising that mixtures of proteins, with their large number of reactive groups, behave somewhat differently from the individual components of the mixture and are difficult to separate.

Serum mucoid is prepared by coagulating serum or even whole blood by heating and precipitating the filtrate with alcohol after dialysis. A yield of 0·1–0·5 g. per litre of blood is obtained by this method. If, as seems possible, the carbohydrate content of serum albumin (about 2·5 % g.-m.-g.) is due to admixture with serum mucoid (containing about 25 % g.-m.-g.), there must be in each litre of serum say 25 g. of albumin and 2·2 g. of serum mucoid, 90 % of which is carried down by the albumin when it is coagulated by heating. It is worthy of note that Levene & Mori [1929] conclude that the carbohydrate content of egg albumin is due to admixture with ovomucoid.

It is certain that the second fraction present in serum albumin presents certain similarities to the protein known as serum mucoid but it is immaterial for the moment whether the two are identical. The important fact established is that there is present in the albumin fraction of serum considerable quantities of some other protein, perhaps amounting to as much as one-tenth of the total albumin fraction.

SUMMARY.

1. Serum albumin, as ordinarily prepared, is apparently not a single protein but is contaminated with varying amounts of a more soluble fraction.

2. By careful fractionation horse serum albumin has been separated into two widely different fractions:

(*a*) Crystalline albumin free from carbohydrate and with a tryptophan content corresponding roughly to one molecule of this amino-acid in each molecule of protein;

(*b*) A freely soluble fraction which is not coagulated readily on heating. This fraction contains considerable amounts of carbohydrate, has a higher tryptophan content than the albumin fraction, has a lower rotatory power and a lower nitrogen content and has fewer free amino-groups.

The author is indebted to the staff of these laboratories for their technical co-operation.

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CCCXIII. IRRADIATION OF FATS.

II. SOME OBSERVATIONS ON METHODS OF ANALYSIS OF OXIDIZED FATS AND ON THE INTERRELATION OF THE RESULTS OBTAINED.

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In any attempt to study the oxidative effect of irradiation of fats, it is obvious that the methods of analysis employed must play an important part. The tests which may be applied to oxidized fats are characterized by lack of precision as regards both the actual quantitative expression and the knowledge of the chemistry involved. Reference to the paper by Lampitt *et al.* [1935] will show that three methods were selected to illustrate the application of the irradiation technique which was described. These methods had been studied with the object of exact standardization of procedure and an early communication on the subject was confidently predicted. Since the publication of the paper in 1935, however, further investigation has resulted in the application of the Zeiss photometer to the measurement of the Kreis test intensity, and in the development of a modification of the Issoglio determination, which yields figures very different from those usually obtained. The present paper details the results of this work, together with certain references to the aldehyde value [Lea, 1934] and the peroxide value [Lea, 1931].

Many workers have indicated the complexity of the changes which take place when comparatively simple chemical substances are irradiated and it can be assumed that the changes are even more complicated in the case of fats. Much has been written in an endeavour to explain and interpret the results of chemical examination of oxidized fats but such speculations cannot be regarded as being really profitable with the meagre data available. The authors have carried out certain work in an endeavour to correlate the results obtained on a series of fats using the tests mentioned. These results are recorded and certain tentative conclusions have been drawn regarding the interrelationship of the substances determined by the various methods.

EXPERIMENTAL.

1. *The Kreis test.*

The test, as modified by Kerr [1918], is capable of detecting incipient rancidity, and the work of Powick [1923] would appear to prove that the red colour produced in the reaction is due to the presence of epihydrinaldehyde in oxidized fats.

Attempts to express the results quantitatively cannot be considered to be entirely satisfactory, although any one of the proposed methods in the hands of the same investigator is probably sufficiently accurate to give comparative results. Methods of quantitative expression already proposed include:

(1) Matching by means of a colour standard (such as potassium permanganate [Pritzker & Jungkunz, 1927]).

(2) Diluting the fat with a neutral substance (such as light petroleum) until the colour obtained matches a standard colour (such as methyl red at pH 4·8 [Holm & Greenbank, 1923]).

(3) Diluting the fat with light petroleum until it only just produces a positive reaction [Kerr, 1918].

Täufel & Sadler [1934] have recently proposed the removal of the reacting substance from the fat in a stream of nitrogen and its subsequent colorimetric determination, using the Kreis test by comparison with known amounts of epihydrinaldehyde.

The objection to the use of artificial colour standards is the fact that they do not always match the Kreis test colour exactly, and a method has therefore been worked out by the authors for the quantitative expression of the Kreis test by means of the Zeiss photometer, the use of which offers the advantages that the personal element is eliminated from the determination and the use of artificial colour standards is avoided.

(*a*) *Purification of reagents.* In the Kreis test, purity of the reagents is an important matter if consistently reproducible colour shades are to be obtained. This applies particularly to the dilution method where a small error in the determination of the point at which a positive test is obtained causes an apparently large alteration in the dilution figure. It has therefore been found necessary to purify the methylated ether for use in the Kreis test and also, at times, the light petroleum.[1]

Hydrochloric acid should be of A.R. quality and colourless. It is preferably stored in the dark and should be discarded as soon as it develops any colour.

(*b*) *The dilution method.* A light petroleum solution of the fat of suitable strength is prepared. A series of test-tubes of identical shape and colour is used, and varying amounts of the solution are measured into the tubes, sufficient light petroleum being added to adjust the volumes to 2 ml. The Kreis test is applied and the dilution at which a negative test is first obtained is used as a measure of the Kreis test intensity.

Such a method of determination is obviously subject to a "personal factor", but it is also greatly affected by the colour of the blank solution. If the latter is coloured (owing to unsatisfactory reagents) the point at which a positive test can be detected is materially altered, and the sensitivity of the test is very much reduced. Although such a method is satisfactory for the comparison of fats which are to be examined together, it can hardly be considered as a suitable method of expressing the Kreis test quantitatively.

(*c*) *Photometric method of quantitative expression of the Kreis test.* In the experiments described herein the technique used has been as follows.

5 ml. of a fat solution in light petroleum are placed in a small separating funnel with 10 ml. concentrated hydrochloric acid and shaken for 2 min. 5 ml. of 0·1 phloroglucinol reagent are then added, and the mixture is shaken for 2 min. The emulsion is run into a 30 ml. centrifuge-tube and whirled (2300 r.p.m.) for 10 min. The volume of the lower acid layer has been found to be 12·5 ml.

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[1] Purification of the ether is effected in a manner similar to that advocated by Werner [1933], by shaking 100 ml. with 20 ml. silver nitrate solution (10 %) and 20 ml. potassium hydroxide solution (5 %) in a separating funnel. After standing some time, with occasional shaking, the lower layer is removed, and, after washing with water, the ether is filtered. The clear ether is dried over fused calcium chloride and filtered. It is then suitable for use in the preparation of the 0·1 % solution of phloroglucinol.

When necessary, light petroleum (B.P. 40–60°) is purified by refluxing with concentrated sulphuric acid, removing the acid by washing and drying with fused calcium chloride.

By means of a dry pipette sufficient of the acid layer is transferred to a 2·0 cm. Zeiss photometer cell,[1] and the colour is measured in the instrument against water as a standard, using a suitable filter. A combination of the Wratten mercury green line filter and a didymium filter (No. G 555*a* Corning Glass Works) has been found to be satisfactory. The limits of the light band transmitted by the combination were sharply defined at 5400 Å. and less sharply at 5550–5600 Å.

A blank determination is made on the reagents using 5 ml. light petroleum instead of the 5 ml. fat solution, and the Kreis test is calculated by use of the formula:

$$\frac{\log B - \log R}{X},$$

where X = wt. of fat in the 5 ml. light petroleum solution, R = photometer reading of the fat solution and B = photometer reading of the "blank" determination.

Table I shows the results obtained by taking two or more different weights of fat in each of a series of seven irradiated fats and demonstrates the accuracy obtainable. Actually, as the result of a large number of such determinations it can be said that the Kreis test intensity figures are reproducible with an error which is not more than 5%. Values obtained by this method have been compared with those obtained by the dilution method, the accuracy of which is

Table I. *Kreis test intensities determined on different weights of butter fat.*

Sample No.	Weight of butter fat in 5 ml. light petroleum solution (g.)	Kreis test intensity
1	0·714	0·30
	0·571	0·30
2	0·453	0·61
	0·272	0·63
3	0·204	1·40
	0·163	1·40
4	0·107	2·28
	0·064	2·32
5	0·077	5·08
	0·062	4·91
6	0·095	5·68
	0·076	5·61
	0·046	5·60
7	0·042	7·32
	0·025	7·56

Table II. *Comparison of Kreis test intensities by the dilution method and the photometric method.*

% oxidized butter fat in fat mixture	Determined Kreis test intensity		Calculated Kreis test intensity for oxidized fat	
	Dilution ratio	Photometer method × 10^3	Dilution ratio	Photometer method × 10^3
100	3100	2090	3100	2090
82·7	1900	1730	2300	2090
66·2	2000	1390	3020	2100
42·1	1000	910	2380	2160
19·7	400	420	2030	2130

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[1] In using the glass cell supplied with the instrument it is necessary to smear the cell-cover with "rubber-grease" before it is placed in position. This effectively seals the cell and prevents the exit of acid fumes.

necessarily much less. Table II gives a series of such figures, obtained for different dilutions of oxidized butter fat with fresh butter fat.

The Kreis test intensities by the Zeiss photometer method are shown to be independent of the weight of fat taken for the test, and the greatest error recorded is 2·5 %. The dilution method is shown to be subject to an error of ± 20 %.

2. *The peroxide value.*

The accurate determination of peroxide in fats has been described by Lea [1931]. The following description of the method includes slight manipulative improvements introduced by the authors.

1 g. fat (or less if the peroxide value is high) is weighed into a pyrex boiling-tube, diameter about 20 mm. which is protected from light by means of a brown paper tube. 1 g. powdered A.R. potassium iodide is added and a mixture of 6·5 ml. A.R. chloroform and 13 ml. A.R. acetic acid. A steady stream of nitrogen is passed into the air space above the liquid by means of a narrow glass tube which is widened for about 2 in. at the lower end. The tube passes loosely through a hole in a rubber stopper (which fits into the pyrex tube) and the upper end carries a glass tap. After 3 min. the stream of nitrogen is stopped by means of the tap, and after removal of the brown paper cover, the tube is immediately heated over a small Bunsen flame until the liquid is boiling freely. The tube is then plunged into a boiling water-bath until the boiling liquid has risen to within half an inch of the rubber stopper. The narrow glass tube is then pulled up until its lower, widened portion closes the hole in the stopper. The closed boiling-tube is now shaken vigorously for a few seconds and plunged into ice-cold water for about a minute. The titration is carried out according to Lea's instructions[1] and the number of ml. of $N/500$ sodium thiosulphate required by the iodine liberated from 1 g. fat must be multiplied by 1·6 to give mg. O_2 per 100 g. fat. This method of presenting the results has been adopted in this paper. The greatest precaution should be taken to protect the fat and reagents from the action of direct light.

In the case of fats which have a high peroxide content, it has been found necessary to use less than 1 g. fat, and this has been done when the titration exceeds about 10 ml. The necessity for this precaution is illustrated by the results given in Table III.

Table III. *Peroxide values determined on different weights of fat.*

	Weight of fat taken (g.)	Peroxide value
Ox kidney fat	0·200	63
	0·249	61
	0·507	52
	1·004	46
Butter fat 1	0·050	90
	0·112	94
	0·203	83
	0·512	71
	1·004	59
Butter fat 2	0·049	166
	0·110	162
	0·226	136
	0·551	118
Butter fat 3	0·062	196
	0·102	197
	0·231	160
	0·442	144

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[1] The end-point is not good, but with a little practice the titration can be made without difficulty.

3. *The Issoglio value.*

(*a*) *Kerr's method.* Apart from a few minor points the following instructions for the determination of the Issoglio value follow the method devised by Kerr [1918].

12·5 g. fat and 50 ml. distilled water are placed in a 300 ml. pyrex flask, and heated on a steam-bath (protected from direct light) for 2 hours. At intervals of 15 min., the contents of the flask are swirled. After 2 hours the mixture is filtered through a wet filter-paper (Whatman No. 42 11 cm.) into a 50 ml. graduated flask and, after cooling, the filtrate is made up to 50 ml. with distilled water. 10 ml. of this solution are mixed with 10 ml. of 20 % sulphuric acid in a 300 ml. pyrex flask, and 50 ml. $N/100$ potassium permanganate solution are added. The solution is heated to boiling, the flask being covered by a small watch glass, and the boiling is continued for exactly 5 min. 50 ml. $N/100$ ammonium oxalate solution are added, and the solution is titrated with $N/100$ potassium permanganate at about 60°.

The Issoglio value is calculated from the formula

$$(t_1 - t_2) \times 3{\cdot}2,$$

where t_1 = No. of ml. $N/100$ potassium permanganate used for oxidizing 10 ml. of filtrate (i.e. extract from 2·5 g. fat), and t_2 is a blank titration obtained in a similar manner on 10 ml. distilled water which has been passed through a wet No. 42 11 cm. filter paper (an average figure is 3·5 ml.).

The Issoglio value is therefore the oxygen equivalent in mg. of the aqueous extract from 100 g. fat.

(*b*) *Method of boiling with potassium permanganate.* In order to ensure identical treatment during the boiling with potassium permanganate, the following procedure has been adopted. Two Bunsen burners and tripods are used; one burner is used for bringing the solution to the boil, and the other is so adjusted that it is sufficiently hot to keep the solution boiling. A clean glass bead is placed in the liquid in order to prevent overheating. The flask containing the permanganate etc. is placed on the first tripod until it begins to boil, and it is then quickly transferred to the second tripod and allowed to boil for exactly 5 min. The adjustments of the Bunsen burners are not altered during the course of a determination, and in this way the blank and the determination receive exactly the same treatment.

(*c*) *Treatment for fats of high Issoglio value.* It was discovered, during the course of an investigation on the irradiation of butter-fat, that the Issoglio value did not increase to its expected magnitude for the longer irradiation periods. This was considered to be due to the possibility that, for fats of high Issoglio value, only a part of the water-soluble oxidizable substances was determined under the standard conditions of the test, as given in (*a*) and (*b*). This is shown by the following example. An oxidized butter fat having an apparent Issoglio value of 58 was re-examined by making the determination on a mixture of 6·25 g. fat plus 6·25 g. fresh butter fat with an Issoglio value of 2. The result obtained was 38, from which it may be calculated that the true Issoglio value of the oxidized fat was $(38 \times 2) - 2 = 74$.

As a result of a number of determinations it was concluded that, employing the technique in question, this dilution procedure was necessary for fats having an Issoglio value higher than 40, 12·5 g. of the mixture being taken for the determination.

(*d*) *Progressive oxidation during the period of extraction.* The method, as described above, was used for obtaining the results presented in a previous paper [Lampitt *et al.*, 1935] but subsequently indications were obtained that oxidation of the fat occurred during the extraction period. Experiments were therefore made in which two precautions were introduced to limit the possibility of oxidation occurring during the determination; the addition of a condenser and the production of an inert atmosphere by a nitrogen stream playing on the surface of the reaction mixture. Results of preliminary tests are given in Table IV.

Table IV. *Effect of an inert atmosphere during the Issoglio extraction.*

Sample No.	Issoglio value obtained Original method	Condenser alone	Condenser and nitrogen
1	1·4	1·2	1·4
2	20·5	15·5	9·5
3	60*	46*	24·5
4	103*	73*	26
5	158†	130†	44·5

* Original fat diluted with fresh butter fat in the ratio 8 : 17.
† Original fat diluted with fresh butter fat in the ratio 5 : 20.

It would appear therefore that, when an Issoglio determination is made by the original method, further oxidation of the fat occurs during the 2 hours' extraction period and that, when the fat is already appreciably oxidized, the further oxidation is very considerable. No definite explanation can be offered of the apparently anomalous results obtained with Sample No. 1 which had a low Issoglio value. In the case of fresh butter fat, however, it is probable that in the original method the fat is not oxidized beyond the induction period, and the two methods would not then be expected to give substantially different results.

Further evidence that the higher values obtained by the original method are due to oxidation of the fat is afforded by the results of determinations in which the time of aqueous extraction was varied. The determinations were made on 2·5 g. fat plus 10 g. fresh butter fat (of known Issoglio value), the results being calculated by the method already indicated. From these determinations which are given in Table VII (original method), it appears that the results obtained by the original method have no quantitative significance, except as empirical values, obtained by adherence to a strict procedure.

(*e*) *Effect of stirring during extraction.* Another aspect of the method which was obviously capable of improvement was the periodic shaking of the fat and water during the 2 hours' extraction, and in this connexion a series of experiments has been carried out in which the periodic shaking was replaced either by gas bubbling or by mechanical stirring. The results are collected in Table V.

From these figures it appears that stirring by nitrogen bubbling is a satisfactory method of ensuring efficient extraction of the fat by the water. The results are definitely higher than when the nitrogen is passed into the flask (and not through the liquid) and they are also slightly higher than those obtained by mechanical stirring. The possibility that oxygen (or some other impurity) in the nitrogen was the cause of these higher results appears to be excluded by the experiments carried out with hydrogen, with washed nitrogen and with nitrogen which had been passed over heated copper. The lower results obtained by

Table V. *Effect of stirring during the Issoglio extraction.*

Sample No.	Nitrogen on surface	Stirring by gas bubbling: Nitrogen	Stirring by gas bubbling: Nitrogen water-washed	Stirring by gas bubbling: Nitrogen over heated copper	Stirring by gas bubbling: Hydrogen	Mechanical stirring: Rotary	Mechanical stirring: Up and down
1	10	14	—	—	—	—	—
2	13·5	18·5	—	—	—	—	—
3	—	17	—	—	—	13·5	—
4	—	17·5	—	—	—	16	16
5	—	33	—	—	—	31	—
6	—	18	17·5	—	—	—	—
7	—	19	—	19·5	—	—	—
8	—	20·5	—	—	20	—	—

mechanical stirring are probably due to the fact that the stirrer was small (since it had to be inserted through the neck of the flask) and, for this reason, not sufficiently efficient.

The use of a stream of nitrogen for stirring the fat and water during the 2 hours period is therefore recommended as an improvement in the method. The results so obtained are very different from those furnished by the original method, but the present authors consider that they are more likely to have some chemical significance.[1]

In the above experiments the nitrogen stream was adjusted to about 6 l. per hour, but no attempt was made exactly to standardize the speed. This point seemed to be worthy of further examination, and experiments were therefore made in which the rate of flow of the nitrogen was different for determinations which were otherwise identical. The actual speeds were not accurately determined, but they were roughly 6 and 12 l. per hour, and it will be seen from the results given in Table VI that the magnitude of the Issoglio determination was not affected by the rate of flow of the nitrogen.

Table VI. *Effect of speed of nitrogen stream.*

Sample No.	6 l. per hour	12 l. per hour
1	17·5	18
2	34	34
3	41	41·5

In view of the increasing values obtained by the original method with increasing times of aqueous extraction, a similar series of Issoglio determinations was made on the same fat by the new method. The results are given in Table VII.

Table VII. *Effect of increasing period of extraction.*

	1 hour	2 hours	3 hours
Original method	57·5	122	194
New method	36·5	43·5	43·5

It is seen that the figure obtained by the new method after 3 hours is the same as that for 2 hours, and it may be concluded that the extraction is complete in the normal time, viz. 2 hours.

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[1] It is obvious that the composition of any products carried over by the nitrogen stream may be of importance. This matter is receiving attention, but it is significant to record that the solution which is obtained by passing the gas through water in a sintered glass bubbler contains substances which are oxidizable by permanganate.

(*f*) *Recommended procedure.* As an outcome of this investigation it is recommended that the Issoglio value should be determined as follows.

12·5 g. fat and 50 ml. of boiled and cooled distilled water are placed in a 175 ml. flask provided with a single-surface condenser having a ground glass joint. A slow stream of nitrogen is bubbled through the contents of the flask by means of a glass tube passing down the inside of the condenser, and the flask is heated on a boiling water-bath for 2 hours in a location protected from direct light. After this time, the tube is raised so that the nitrogen no longer bubbles through the liquid, and the flask is allowed to remain on the water-bath for a few minutes until the fat layer has separated. The contents of the flask are then filtered through a wet filter-paper (Whatman No. 42 11 cm.) into a 50 ml. graduated flask, and after cooling, the volume of the filtrate is made up to 50 ml. with distilled water. The permanganate equivalent of this solution is determined as previously described.

It is permissible to make determinations on less than 12·5 g. fat, provided that the weight taken is increased to 12·5 g. with fat of known Issoglio value. In this case, the Issoglio value will be given by the formula

$$b + 12{\cdot}5\,(c-b)/a,$$

where a = weight of fat taken, b = Issoglio value of fat used for dilution, c = Issoglio value determined on the mixture.

The results of such determinations made on five irradiated butter fats are given in Table VIII. The fresh butter fat used for the dilutions had an Issoglio value of 1·55.

Table VIII. *Issoglio values determined on varying amounts of original fat diluted to 12·5 g. with fresh butter fat.*

Sample No.	Weight of original fat (g.)								
	12·5	8·7	7·5	7·0	6·5	6·25	5·0	2·5	2·0
1	6·0	—	—	6·0	—	—	—	—	—
2	19	—	—	—	19	—	—	—	—
3	32	—	—	—	—	—	30	—	—
4	44	43	44	—	—	—	42	44	—
5	100	—	—	—	—	113	—	—	110

4. *The aldehyde value.*

The determination of "aldehydes" in oxidized fats by formation of the bisulphite compounds was proposed by Lea [1934] and his method has been employed by the present authors without serious modification. Although the end-points of the titrations are somewhat difficult to judge, it is considered that the determination affords an approximate indication of the "aldehyde" content of an oxidized fat. Experience gained during the course of these investigations would indicate that the results are subject to a maximum error of ± 15 %, although the results of duplicate determinations usually agree more satisfactorily. The factors responsible for the erratic results which are sometimes obtained have not been investigated, but the fundamental weakness of titrating sulphite with iodine in bicarbonate solution is a possible explanation. Since the identity of the substances determined is not known, the results have been expressed as (CO) in parts per million, i.e. 1 ml. $N/500$ iodine per g. fat = 28 parts per million of (CO).

5. *Application of the tests described to the examination of irradiated butter fats.*

The method adopted in this phase of the work has been to determine the peroxide value, the Kreis test intensity, the aldehyde value and the Issoglio value on eight samples of the same butter fat which had been oxidized to different extents by irradiation under the mercury quartz lamp. In addition, the first three of these determinations were also made on the fats remaining after the Issoglio extraction had been made (designated "Issoglio fat" in Table IX). In the latter cases, the fat was filtered through a dry fluted No. 41, 9 cm. Whatman filter-paper, without further heating and without undue exposure to light. Table IX gives the results which have been obtained.

Table IX. *Analytical results obtained on irradiated butter fat.*

		Peroxide value			Kreis test intensity			Aldehyde value		
Sample No.	Issoglio value	Original fat (*a*)	Issoglio fat (*b*)	Ratio $(a-b)/a$	Original fat (*c*)	Issoglio fat (*d*)	Ratio $(c-d)/c$	Original fat (*e*)	Issoglio fat (*f*)	Ratio $(e-f)/e$
1	4·0	16	17·5	—	0·22	0·11	0·50	40	35	0·13
2	11·0	39	34	0·13	0·58	0·29	0·50	125	65	0·48
3	17·0	68	55	0·19	1·51	0·72	0·52	160	70	0·56
4	—	74	—	—	1·73	—	—	160	—	—
5	28·5	114	96	0·16	3·31	1·38	0·58	265	115	0·57
6	29·5	119	108	0·09	3·85	1·88	0·51	280	130	0·54
7	38·0	132	108	0·18	3·92	1·67	0·57	290	115	0·60
8	—	144	—	—	4·58	—	—	305	—	—
9	—	158	—	—	6·18	—	—	360	—	—
10	43·5	194	151	0·22	6·12	2·56	0·58	465	215	0·54
11	—	195	—	—	6·82	—	—	400	—	—
12	51·5	210	174	0·17	8·33	3·48	0·58	465	195	0·58

DISCUSSION.

Although no definite conclusions can be drawn from these results, there are several points of interest to which attention may be called.

(*a*) It would appear to be significant that in the case of eight fats, varying widely in degree of oxidation, the pretreatment of the fat by the modified Issoglio technique results in a reduction of the peroxide value by about 16%, of the Kreis test intensity by about 55% and of the aldehyde value by about 55% (excluding sample No. 1). This would appear to indicate an equilibrium distribution of the various substances between the aqueous and the fat layers during the Issoglio determination. This is not surprising in the case of the last two determinations, but in the case of the peroxide value it is somewhat difficult to understand. If the peroxides were decomposed by water during the 2 hours' extraction, one would expect a greater reduction than one-sixth, and it is difficult to imagine that they are extracted in any strict sense of the word. It is possible, however, that during the extraction period the peroxides react with oxidizable substances (for example, aldehydes) present in the fat, and that their disappearance is to be accounted for in this manner. A further possibility is that two types of peroxide exist in irradiated fats, only one of which is decomposed under the conditions obtained in the Issoglio extraction.

(*b*) It is perhaps remarkable that the relative reduction in the aldehyde value should in all cases be practically identical with the relative reduction in the Kreis test intensity; in other words, the extraction by water in the modified

Issoglio determination results in the removal of 55% of the bodies giving the Kreis test and 55% of those determined as aldehydes. This would suggest some close relation between the two determinations, but the nature of this relation is not apparent from the results of the determinations on the original fats.

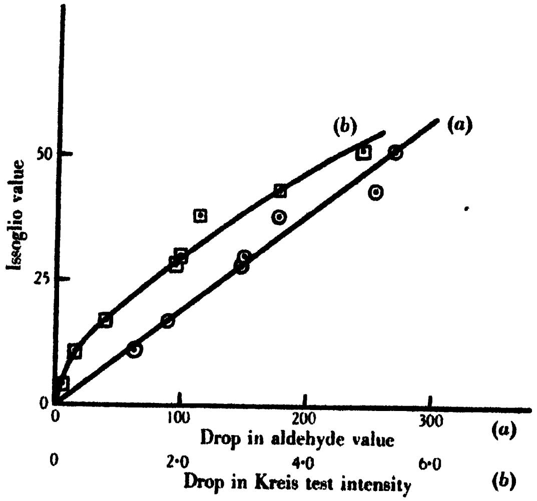

Fig. 1.

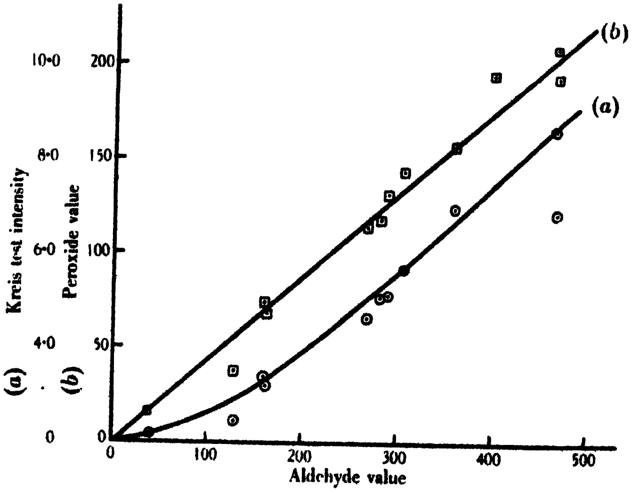

Fig. 2.

The Kreis test is associated with epihydrinaldehyde, or with a compound which can give rise to epihydrinaldehyde, and it is of course possible that this substance will be determined and included in the aldehyde value. However, it would appear from Fig. 2 that the magnitude of the Kreis test does not bear any simple relation to the total aldehydes, and it may be concluded, at least tentatively, that the Kreis test substance does not constitute a fixed proportion of the determined aldehydes.

(c) The determinations presented above were carried out with the intention of elucidating the nature of the substances determined in the Issoglio value. It was thought that perhaps the Issoglio value might be a composite determination including definite contributions from the aldehyde value, the peroxide value and the Kreis test, and that it might be possible to discover some simple relations between these four determinations. This object has not been definitely achieved, but Fig. 1 shows the relation between the Issoglio value and the reductions in the Kreis test intensities and also a similar curve for the reductions in the aldehyde values.

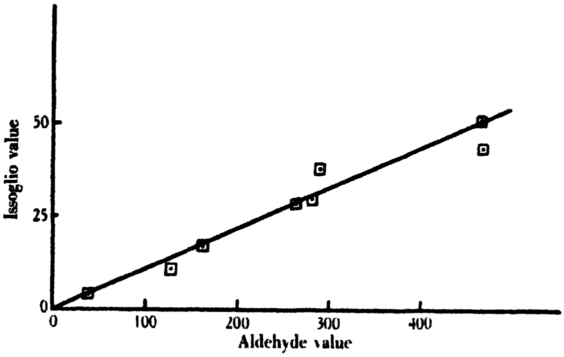

Fig. 3.

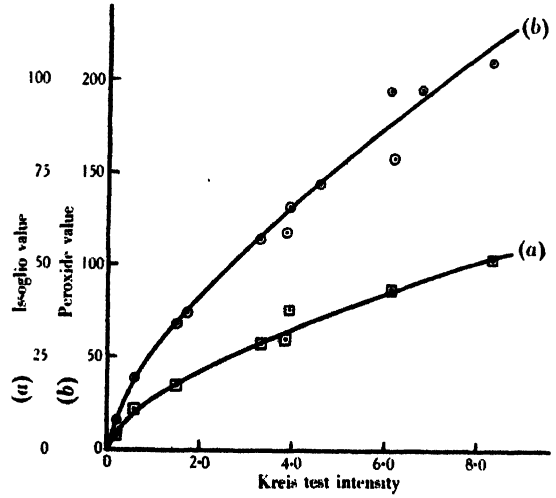

Fig. 4.

In the former case, there is obviously no proportionality, but in the case of the aldehyde value, the results are certainly suggestive, especially when consideration is given to the magnitude of the errors inherent in the three determinations involved.

A similarly linear curve is of course also obtained when the Issoglio value is plotted against the original aldehyde value (Fig. 3), since the Issoglio extraction results in the removal of a roughly constant proportion of the aldehydes.

In Fig. 5 the Issoglio value is plotted against the peroxide value and again the curve appears to be a straight line. This is contrary to the authors' previous suggestion [Lampitt *et al.*, 1935], but it should be remembered that the present Issoglio values have been determined by the new method. It will be seen also that a straight line relation appears to hold between the Issoglio values and the reductions in the peroxide values.

Fig. 4 shows that the Kreis test does not vary proportionally with either the peroxide value or the Issoglio value, and this is in accord with observations previously recorded. Similarly there is no proportionality between the Kreis test and the aldehyde value (Fig. 2). Fig. 2 shows also the relation between the aldehyde value and the peroxide value, and the curve appears to be practically a straight line.

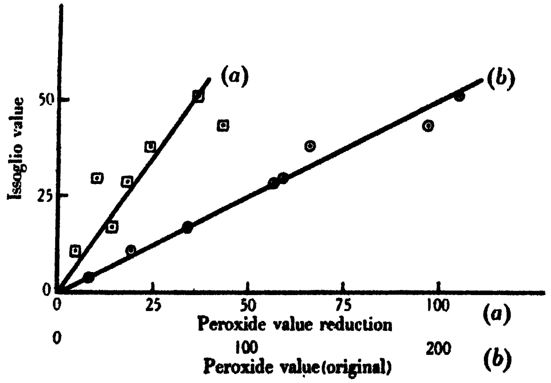

Fig. 5.

A tentative conclusion is that the aldehyde value and the peroxide value increase in rough proportionality and that, since the Issoglio value determines about 55 % of the aldehyde value, it is therefore also proportional to the peroxide value.

SUMMARY.

1. A method of evaluating the intensity of the Kreis test colour by the use of the Zeiss photometer has been presented.

2. An improved method of determination of the Issoglio value has been described, the chief modification being the use of a stream of nitrogen for stirring the fat during the period of aqueous extraction.

3. A technique for the determination of peroxides by Lea's method has been described, and it is shown that the weight of fat should not be greater than the amount required to give a titration of about 10 ml. of $N/500$ sodium thiosulphate.

4. Figures are given for determinations of Kreis test intensities, peroxide values and aldehyde values on irradiated fats both before and after the Issoglio determination. The latter has been found to cause a reduction of about 16 % in the peroxide value, about 55 % in the Kreis test intensity and about 55 % in the aldehyde value. These figures were not dependent on the degree of oxidation of the fats.

5. The Issoglio value appears to be proportional to the reduction in the aldehyde value and also to the aldehyde value of the original fat.

6. The aldehyde value and the peroxide value have been found to be roughly proportional. Since the Issoglio value is proportional to the aldehyde value, it is also porportional to the peroxide value.

The authors wish to record their thanks to Messrs J. Lyons and Co., Ltd., in whose laboratories this work was carried out, for permission to publish, and also to Miss D. A. Rudland, and to Mr A. N. Ainsworth, for their assistance in the practical work.

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CCCXIV. OESTRIOLGLUCURONIDE.

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(*Received 9 October 1936.*)

IN a previous communication [Cohen & Marrian, 1936], the isolation of a water-soluble substance containing a high proportion of combined oestriol was reported. Although no great confidence in the purity of this substance was felt, since it could not be isolated in a crystalline form, the suggestion was made on the basis of carbon and hydrogen analyses and certain colour reactions that it was an oestriolglucuronide. The method described for the isolation of this substance was long and tedious and the yields obtained were poor. Owing to the small amounts available it was not possible to study the properties of the substance in detail or to determine its physiological potency.

The method which has now been adopted for the isolation of the compound, although essentially similar to that originally described, is considerably shorter and results in excellent yields of a product of high purity. The steps in this modified method are shown in Table I. Attention may be drawn to the following points: (*a*) The neutralization of acid butanol extracts with ammonium hydroxide instead of sodium hydroxide. In this way high alkalinity of the evaporating extracts is more easily avoided. (*b*) Elimination of the extractions with 90 % ethanol and 92 % pyridine. Extraction of aqueous solutions with a 1:2 mixture of pyridine and benzene was used with some batches and was found to be a valuable method of purification in cases where the quantity of glucuronide in the urine was small. Good yields of a pure product, however, have been obtained without its help. (*c*) Elimination of the process in which a butanol solution was washed with 0·8 % sodium carbonate and then extracted with *N*/50 sodium hydroxide. In the present work it was found that the carbonate washing of the butanol caused very great losses of glucuronide. It is difficult to understand why this process gave such excellent results in the original work. (*e*) Crystallization of the final product as the sodium salt from methanol.

The crude products yielded by this process were recrystallized several times from methanol. When pure anhydrous methanol was employed, crystals melting at about 305° and analysing excellently for $C_{24}H_{31}O_9Na, 0{\cdot}5\,CH_3OH$ were obtained. Methanol containing small amounts of water yielded a crystalline product melting at about 256° and analysing for $C_{24}H_{31}O_9Na, 1{\cdot}5\,H_2O$.[1] The higher-melting product was found to be converted into the lower-melting one by treatment with water, while the reverse process could be effected by repeated crystallization from anhydrous methanol. It was not possible to remove either the methanol or water of crystallization by heating *in vacuo* at 80° over phosphorus pentoxide.

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[1] It should be pointed out that owing to the decomposition that occurs at and just below the melting point in all these compounds, the exact thermometer reading varies considerably according to the rate of heating. Differences of 1 or 2° in the melting points recorded for different preparations have therefore little significance.

Table I.

Urine
Concentrated to approx. 1/8 vol. Acidified to pH 2·5–3·0, saturated with NaCl and extracted with BuOH.
→ *Urine*

BuOH extract
Concentrated to 1/2 vol. and extracted with $N/3$ NaOH.
→ *BuOH*

$N/3$ NaOH extract
Acidified to pH 2·5–3·0, saturated with NaCl and extracted with BuOH.
→ *Aqueous solution*

BuOH
Neutralized with NH_4OH and evaporated to dryness. BuOH residue dissolved in H_2O and extracted with ether.
→ *Ether*

Aqueous solution
Extracted with quinoline
→ *Aqueous solution*

Quinoline
Washed with 10% Na_2CO_3.
→ *10% Na_2CO_3*
Extracted with $N/10$ NaOH.
→ *Quinoline*

$N/10$ NaOH
Acidified to pH 3·0 and extracted with BuOH. BuOH extract washed with water and evaporated to dryness.

BuOH residue
Dissolved in absolute methanol and pH of solution adjusted to 7·0–7·5 by addition of methanolic NaOH. Solution concentrated to small volume under nitrogen.
↓
Sodium salt of oestriolmonoglucuronide.

Acidification of aqueous solutions of the sodium salt yielded the free glucuronide in the form of a gel. Solid but amorphous preparations were obtained by dissolving the gel in alcohol and evaporating to dryness. Different preparations had sharp melting-points ranging from about 196° to 236°. The different melting-points appeared to be caused by varying amounts of water of "crystallization". In the case of one preparation drying at 80° *in vacuo* over P_2O_5 removed all the bound water, yielding a product which analysed correctly for the anhydrous glucuronide. With other preparations, however, it was not possible to obtain a completely anhydrous product by drying even at 140°. The reason for these differences is obscure and, since the question is of no great importance, the experimental results are not reported in full.

The crystalline sodium salt gave a strongly positive naphthoresorcinol test, a negative Benedict test before and a positive Benedict test after hydrolysis with dilute acid. Like most of the naturally occurring glucuronides, it is laevorotatory.

Millon's test was strongly positive in the cold. It was previously suggested, tentatively [Cohen & Marrian, 1936], that this might indicate that the phenolic hydroxyl group of the oestriol was not conjugated with the glucuronic acid. Confirmation of this has now been obtained by (*a*) spectrographic and (*b*) chemical methods.

(*a*) It has recently been shown by Callow [1936] that compounds of the oestrin series in which the phenolic group is free show a shift in absorption maximum from 2800 to 2950 Å. in the presence of alkali owing to salt formation. This shift of absorption maximum in alkaline solution was not observed in compounds of the oestrin series in which the phenolic hydroxyl was combined. Callow pointed out that use might be made of this observation in the determination of the structure of naturally occurring conjugated oestrogens such as oestriol glucuronide. Dr Callow has since very kindly examined spectrographically a specimen of the crystalline sodium salt and he has been able to show that in alkaline solution the shift in absorption maximum characteristic of a free phenolic hydroxyl does occur. The authors are grateful to Dr Callow for permission to include his results in the present paper.

(*b*) The glucuronide was methylated with methyl sulphate and alkali and the crude product hydrolysed with dilute acid. There was isolated from the hydrolysate a pure crystalline product which proved to be oestriol monomethyl ether. This provides the first strictly chemical evidence that oestriol is present in the complex, and since in oestriol monomethyl ether the phenolic hydroxyl is not free, it provides further proof that the phenolic hydroxyl group of oestriol is free in the glucuronide.

It was previously stated by Cohen *et al.* [1935] "that the precursor present in urine which gives rise to ether-soluble oestrin on treatment with acid has only a low oestrogenic potency when tested on ovariectomized mice". The significance of this fact in connexion with the assay of oestrin in urine was emphasized and attention was drawn to its possible physiological importance. Since the above statement was based on biological assays carried out with whole pregnancy urine, it was obviously of importance to determine the potency of the pure oestriol-glucuronide. Assays on ovariectomized adult mice by the method of Marrian & Parkes [1929] showed that the glucuronide had a potency of 370 m.u. per mg. Simultaneous tests with oestriol by an identical procedure showed a potency of 10,700 m.u. per mg. The oestriol in the glucuronide has therefore only about 1/17 of the potency of the same amount of oestriol in the uncombined state when assayed by the technique described.

An exact comparison of the potencies of oestriol and its glucuronide is not possible since, as can be seen in Fig. 2, the slopes of the two dose-response curves differ considerably and it seems likely therefore that the ratio of the potencies of the two compounds would vary significantly according to the method of assay. No marked differences could be observed in either the time of onset or the duration of the vaginal responses induced by the two substances.

EXPERIMENTAL.

Isolation of the sodium salt of oestriolglucuronide (batch 9).

30 litres of urine collected from women during the last 2 months of pregnancy were concentrated under reduced pressure at 70–80° to about 4 litres. The mixture was saturated with sodium chloride, acidified to *p*H 2·5 and extracted seven times with 1 litre volumes of butanol. Troublesome emulsions were broken by centrifuging. The combined butanol extracts were made slightly alkaline with NH_4OH, concentrated under reduced pressure to about 3 litres and extracted seven times with 500 ml. volumes of $N/3$ NaOH. The combined alkaline extracts were saturated with NaCl, acidified to *p*H 2·8 and extracted five times with approximately 600 ml. volumes of butanol. The butanol extract was washed

once with saturated aqueous NaCl, made slightly alkaline with NH_4OH and evaporated to dryness. The residue weighed 68 g.

This material was dissolved in 200 ml. of water, and the solution, after acidification to *p*H 3·0, was washed four times with 100 ml. volumes of ether, and then extracted five times with approximately 50 ml. volumes of quinoline. The quinoline extract was washed four times with 50 ml. volumes of 10% aqueous Na_2CO_3. The combined carbonate washings were "back-extracted" four times with 50 ml. volumes of quinoline to minimize losses of glucuronide at this stage. The combined quinoline extracts were extracted with four successive volumes of 100 ml. *N*/10 NaOH, and the combined alkaline extracts, after acidification to *p*H 3·0, were extracted once with 150 ml. and four times with 50 ml. volumes of butanol. The butanolic solution was washed four times with small volumes of water and evaporated to dryness under reduced pressure. The residue weighed 8·5 g. and direct colorimetric assay (without hydrolysis) showed it to contain 1 g. of combined oestriol. It should here be mentioned that colorimetric assays after hydrolysis with acid were conducted at all the preceding stages in the process. These showed that the losses of glucuronide in the discarded fractions were insignificant, but owing to the difficulty of attaining satisfactory hydrolysis, the figures have little real quantitative value and are therefore not reported.

The residue from the evaporation of the butanol was dissolved in about 50 ml. of methanol and after heating nearly to boiling the *p*H of the solution was adjusted to 7·0–7·5 by the careful addition of saturated methanolic NaOH. The solution was then slowly evaporated to about 30 ml. under a stream of nitrogen, by which time a heavy greyish crystalline precipitate had settled out. After chilling, this was filtered off and washed with about 20 ml. of cold methanol. The product weighed 0·654 g. and melted at 264–265° (decomposition and evolution of gas) after preliminary shrinking at 239–240°. No more precipitate could be obtained by further concentrating the filtrate.

The crude crystals were extracted repeatedly with small volumes of hot anhydrous methanol until all but a small amount of dark material was in solution. The combined methanolic extracts were then concentrated slowly under nitrogen to about 60 ml. After chilling, filtering and washing with cold methanol, 0·313 g. of material, M.P. 298–299° (decomposition and evolution of gas) with slight browning at 265–270°, was obtained. After drying at 80° *in vacuo* over P_2O_5, the M.P. was raised to 305–306°. Further concentration of the methanolic filtrate yielded a further 0·15 g. of crystals of the same degree of purity and 0·038 g. with a slightly lower M.P.

Properties of the sodium salt.

Analyses were carried out by Dr Schoeller on material obtained from batch 9, M.P. 305–306°. Found: C, 58·27, 58·19; H, 6·51, 6·43; Na, 4·78%. Calculated for $C_{24}H_{31}O_9Na$, 0·5 CH_3OH; C, 58·57; H, 6·62; Na, 4·58%.

Oestriol content (direct colorimetric assay)—Found: 55·0, 56·7, 58·3%. Calculated for $C_{24}H_{31}O_9Na$, 0·5 CH_3OH: 57·4%.

1·2 mg. gave a positive Tollens's naphthoresorcinol test. 0·21 mg. gave a positive Millon's test in the cold. 0·21 mg. gave a negative Benedict's test. 0·21 mg. after heating at 120° for 1·5 hours with *N*/10 HCl and neutralizing gave a positive Benedict's test.

In the case of certain batches the methanol used for the crystallization was not anhydrous. The product after drying at 80° *in vacuo* over P_2O_5 had M.P. 256–257° (decomposition and evolution of gas) after shrinkage at 242°. (Found (Schoeller): C, 56·33, 56·15%; H, 6·68, 6·68%; Na, 4·32%. Calculated for

$C_{24}H_{31}O_9Na$, 1·5 H_2O; C, 56·14%; H, 6·68%; Na, 4·48%.) After two crystallizations of this material from anhydrous methanol, the M.P. was raised to 301–302°. Mixed with directly isolated material of M.P. 305–306°, the M.P. was 304–305°. Directly isolated material of M.P. 305–306° was dissolved in water and evaporated to dryness under a stream of nitrogen. The product had M.P. 258–259° which was unchanged by admixture with the directly isolated lower-melting compound.

The optical rotation of the sodium salt (M.P. 305–306°) was determined in a Fischer micro-tube. Owing to the opalescence of the aqueous solution, the concentration had necessarily to be low. The observed rotations were therefore small, making very great accuracy impossible. $c=0{\cdot}4966$ (water), $\alpha=-0{\cdot}07°$, $[\alpha]_{5461}^{28°}=-28{\cdot}2°$; $c=0{\cdot}5027$ (water), $\alpha=-0{\cdot}052°$, $[\alpha]_{5461}^{28°}=-21{\cdot}0°$.

Ultraviolet absorption spectrum of oestriolglucuronide in alkaline solution.

The following experiment was carried out by Dr R. K. Callow of the National Institute for Medical Research, London. 5 ml. of a 0·02% aqueous solution of the sodium salt of oestriolglucuronide (M.P. 303–304°) were diluted with 0·5 ml. of water and photographed. A second 5 ml. were diluted with 0·5 ml. of $N/10$

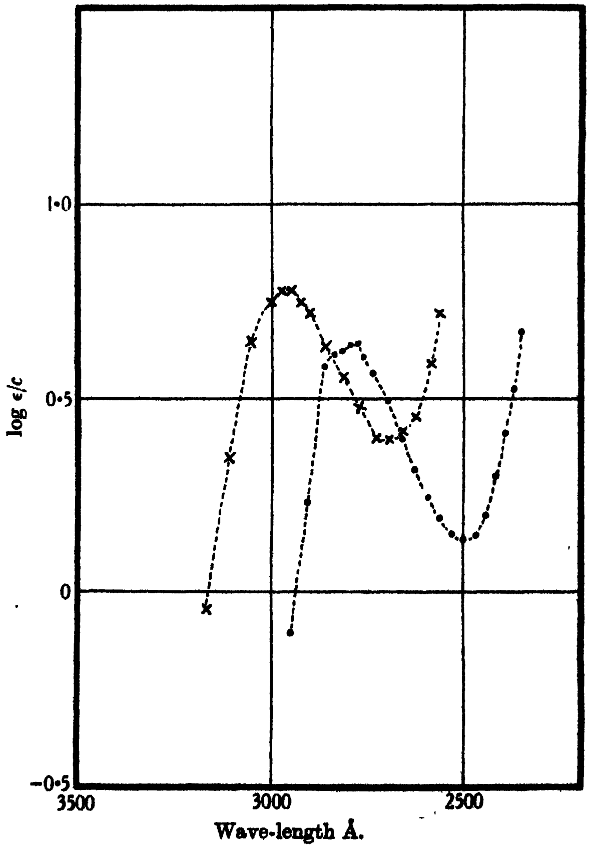

Fig. 1. •----• Oestriolglucuronide, 0·0182% neutral aqueous solution.
×---× Oestriolglucuronide, 0·0182% solution in 0·0091 N NaOH.

NaOH and photographed. The results are shown in Fig. 1 in which log ϵ/c is plotted against wave-length, and where ϵ is the extinction coefficient as defined by the equation:

$$\epsilon=\frac{1}{d}\log_{10}\left(\frac{\text{incident light}}{\text{transmitted light}}\right)-\epsilon_0,$$

and c is the concentration in g. per litre.

The authors are also indebted to Dr Callow for the following information: "...assuming no interference with the absorption of the oestriol part of the molecule, the difference in intensity of absorption between oestriol and its glucuronide corresponds to an oestriol content of 57·5% in the latter." (Calc. for $C_{24}H_{31}O_9Na$, 0·5 CH_3OH: oestriol, 57·4%.)

Isolation of oestriol monomethyl ether from the hydrolysis products of methylated oestriolglucuronide.

171 mg. of the sodium salt of oestriolglucuronide were dissolved in 40 ml. of *N* NaOH and shaken at intervals for 24 hours, methyl sulphate being added in small portions from time to time. No attempt was made to isolate and purify the bulky flocculent precipitate which separated at this stage. A preliminary experiment suggested that hydrolysis at *p*H 1·0 for 2 hours at 120° caused too extensive destruction to permit the isolation of the hydrolysis product in a state of purity. Milder conditions were therefore employed. The whole methylation mixture was acidified with HCl to *p*H 1·5 and heated at 100° for 5 hours. The mixture was cooled and then repeatedly extracted with ether. The ethereal extract was washed several times with *N* NaOH, then with water and finally evaporated to dryness. The product was a dark greenish blue semicrystalline solid and weighed 16·4 mg. The aqueous phase from the ether extraction was combined with the NaOH washings and rehydrolysed for 4 hours at *p*H 1·2. The mixture was worked up as before, yielding a further 24·5 mg. of material similar to that obtained from the first hydrolysis.

The products were combined, boiled twice with charcoal in alcoholic solution, whereby all but a trace of the blue colour was removed, and recrystallized twice from 40% ethanol. The product crystallized in hexagonal plates identical in appearance with authentic oestriol monomethyl ether crystallized from the same mixture. The M.P. was 165–168°, which was unchanged after admixture with authentic oestriol monomethyl ether of M.P. 166–168°.

Biological assay of the sodium salt of oestriolglucuronide.

The method of Marrian & Parkes [1929] was employed. Aqueous solutions of the sodium salt of different concentrations were injected into groups of twenty adult ovariectomized mice. The mice were injected at 9 a.m. and 5 p.m. on the first and second days, the volume of each injection being 0·1 ml. Vaginal smears were taken at 9 a.m., 5.30 p.m. and 11 p.m. on the third and fourth days, at 9 a.m. and 5.30 p.m. on the fifth day and at 9 a.m. on the sixth day. The smears were stained with methylene blue before examination. A smear from which leucocytes were absent and in which cornified cells were present was deemed to be positive. That is to say a smear consisting of mixed nucleated epithelial cells and cornified cells was counted as a positive, while a pure nucleated smear was counted negative. The occasional "doubtfully positive" smears have been counted as half positives. It is necessary to emphasize these points, since it appears reasonably certain that the great differences which have been reported in different laboratories for the potency of oestriol are mainly due to the different criteria which are accepted for a positive vaginal reaction.

Similar tests were carried out at the same time with a highly purified specimen of oestriol. The curves obtained by plotting the percentage positive response of each group of mice against the quantity injected are shown in Fig. 2. The mouse unit is defined as the amount necessary to produce a 50 % response.

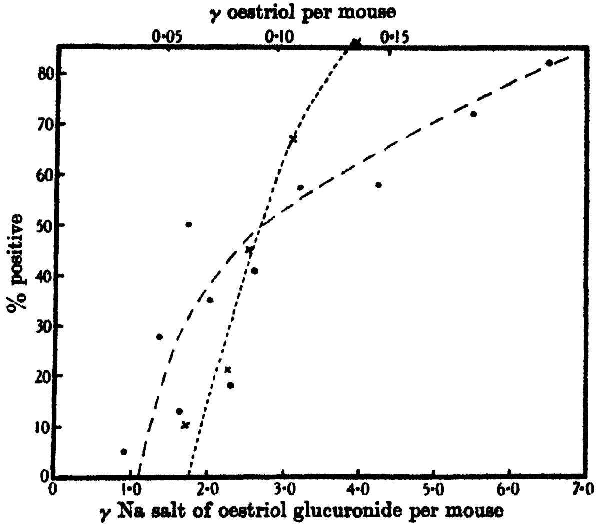

Fig. 2. ×- - -× Oestriol. •——• Na salt of oestriolglucuronide.

Summary.

1. An improved method for the isolation of the crystalline sodium salt of oestriolglucuronide from human pregnancy urine is described. Yields as high as 500 mg. from 30 litres have been obtained.

2. Crystallization of the crude sodium salt from anhydrous methanol yields a product, M.P. about 305° (decomposition and evolution of gas), which gives analytical figures corresponding to a composition $C_{24}H_{31}O_9Na$, 0·5 CH_3OH. Crystallization from methanol containing traces of water yields a product, M.P. about 256° (decomposition and evolution of gas), which analysed to $C_{24}H_{31}O_9Na$, 1·5 H_2O.

3. Spectrographic evidence provided by Dr R. K. Callow confirms the suggestion previously made that the phenolic hydroxyl of the oestriol is free in the glucuronide. Further confirmation of this was provided by isolating oestriol monomethyl ether from the hydrolysis products of the methylated glucuronide.

4. Tested on adult ovariectomized mice, the sodium salt of the glucuronide showed a potency of 370 m.u. per mg. Oestriol tested under the same conditions showed a potency of 10,700 m.u. per mg.

The authors are greatly indebted to Dr M. Watson of the Department of Obstetrics and Gynaecology and to Miss Cassels of the Department of Social Service, Toronto General Hospital, for their co-operation in the collection of urine. They also wish to acknowledge gratefully the help given by Miss Dorothy Skill in much of the experimental work.

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CCCXV. THE EFFECT OF FUMARATE ON THE RESPIRATION OF LIVER AND KIDNEY TISSUE.

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(*Received 15 October 1936.*)

THE interesting theory of Szent-Györgyi and co-workers [Annau *et al.*, 1935] that in respiration the system fumarate-oxaloacetate functions catalytically in transporting activated metabolite hydrogen to the cytochrome (Warburg-Keilin) system has received support in the experiments of Stare & Baumann [1936]. Using the manometric method with pigeon breast muscle and heart muscle (swine), the latter investigators observed the following:

"1. The oxygen uptake of tissues is increased by very small amounts of fumarate and the increase is much greater than can be accounted for by the oxidation of the fumarate itself.

2. Small amounts of added fumarate are detectable after prolonged contact with respiring tissue.

3. The increased respiration is normal, that is, the respiratory quotient is not materially changed by the addition of small amounts of fumarate.

4. Substances which yield fumarate on contact with tissue—succinate, malate, oxaloacetate—show a similar action to fumarate itself."

Recently Szent-Györgyi and co-workers [Annau *et al.*, 1936] have applied quantitative methods for the micro-estimation of oxaloacetic, pyruvic, and malic acids and have given additional evidence in support of their theory. They followed chemically the disappearance of oxaloacetic acid added to muscle and observed a simultaneous increase in malic acid, with only a very small increase in pyruvic acid. They are of the opinion that most of this pyruvic acid comes from the oxidation of a triose or triosephosphate and not from the decarboxylation of the added oxaloacetate. They have also shown chemically that oxaloacetate is formed in muscle tissue from added fumarate. These findings are of fundamental importance since they give chemical indication of the mechanism of the catalytic action of fumarate and related compounds in respiration.

The writer had the opportunity of spending some time in Prof. Szent-Györgyi's laboratory and of repeating and confirming the basic chemical estimations recently given. It is the object of this paper to report on manometric and chemical experiments which show the applicability of the fumarate-oxaloacetate catalytic theory to the respiration of the liver and kidney tissue of the rabbit.

Methods.

The measurements of oxygen consumption were made in Warburg manometers at a temperature of 37° and in a Ringer-phosphate buffer. As previously shown [Stare & Baumann, 1936] this solution is a more satisfactory medium than plain phosphate buffer to demonstrate the activity of fumarate and related compounds.

The rabbits were killed by decapitation. The liver and kidneys were immediately removed, washed briefly in ice cold water, ground in an ice-cold mincer, and suspended in ice-cold Ringer-phosphate buffer in the concentration of 1 g. of tissue to 3 ml. of buffer. 2 ml. of this suspension were pipetted into each Warburg vessel. The volume of the fluid in the respiration vessel was always 4 ml.

For the chemical estimations the tissue suspension was pipetted into small Erlenmeyer flasks and diluted with water and various supplements to the same concentration as used in the respiration vessels. These flasks were agitated in a bath at 37° for periods from 0 to 20 min. and the reactions stopped by the addition of tungstic acid. The contents were then centrifuged and the clear protein-free liquid used for the chemical estimations. The details of the various chemical estimations are given by Straub [Annau *et al.*, 1936]. It should be mentioned that the method of estimating pyruvic acid is affected by acetone. Using the nitroprusside test acetone was estimated and its formation observed to be negligible during the short time of these experiments.

Results and discussion.

Figs. 1 and 2 show the effects of fumarate and malonate on the respiration of liver and kidney tissue. They are representative experiments from a total number of eight for each tissue. The general effects previously described with muscle tissue are observed, malonate inhibits respiration, fumarate "preserves" it, and when both are added the malonate inhibition is overcome and the tissue appears to respire normally. Whether or not fumarate plus malonate shows the same conserving action as fumarate alone depends upon the intensity

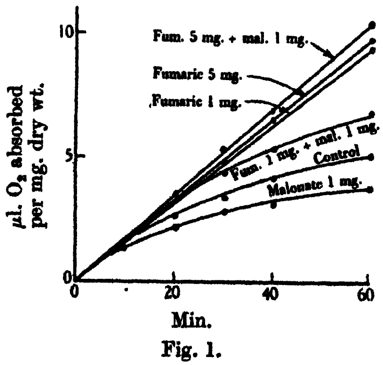

Fig. 1.

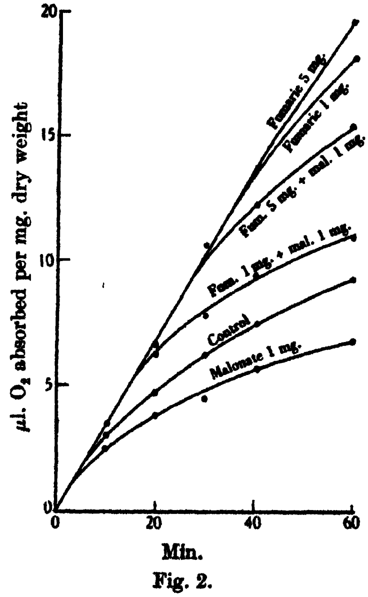

Fig. 2.

Fig. 1. The effects of fumarate and of malonate on the respiration of rabbit liver. (Ringer-phosphate buffer—concentrations of fumarate and of malonate expressed as mg. added to 4 ml. volume in Warburg vessel.)

Fig. 2. The effects of fumarate and of malonate on the respiration of rabbit kidney. (Ringer-phosphate buffer—concentrations of fumarate and of malonate expressed as mg. added to 4 ml. volume in Warburg vessel.)

of the respiration, the duration of the experiment, and the absolute amounts of fumarate and malonate present.

Malonate is known to poison succinic dehydrogenase. As Szent-Györgyi has shown [Annau *et al.*, 1936] a small part of the oxaloacetic acid is reduced to succinate. In the presence of malonate this succinate is not oxidized to fumarate, and respiration is inhibited owing to a lack of essential fumaric acid. The "time" at which malonate inhibits respiration, therefore, depends upon when the available fumaric acid has been converted into succinate. This is dependent upon the intensity of the respiration, the duration of the respiration and the absolute amounts of fumaric and malonic acids present within the cell.

From Figs. 1 and 2 it is observed that without added fumarate, malonate inhibits within the first 5 min. of the experiment, showing that by that time the normal fumarate content is converted into succinate. When 1 mg. each of fumarate and malonate are added respiration proceeds normally for the first 15–20 min. By this time malonate inhibition is observed and it is probable that the added fumarate which has penetrated the cell has been inactivated by conversion into succinate. With larger amounts of fumarate a longer time is required for its conversion into succinate and the consequent appearance of malonate inhibition. Without added malonate, the conserving action of fumarate may be shown within such wide limits as 0·0001 to 0·02 *M*.

The extent of malonate inhibition is dependent to some extent on the amount added. In the experiments reported, a concentration of 1 mg. in 4 ml. was used and an inhibition of 25–30 % obtained. If the concentration is increased to 4 mg. in 4 ml. approximately 50 % inhibition is observed. This is probably a result of maintaining a higher concentration of malonate within the cell and thus more effectively blocking the succinic dehydrogenase.

The total concentration of the various dicarboxylic acids must not be too high or a general non-specific inhibition of respiration is obtained after the first 30–40 min. This appears to apply particularly to kidney tissue which seems to be more sensitive to such changes.

Oxaloacetate is very rapidly reduced by tissue and hence its concentration in the tissue is always low. Yet its formation can be demonstrated by the hydrazine fixation method as described by Banga [Annau *et al.*, 1936]. That liver and kidney tissue form oxaloacetate from fumarate was shown by the following experiment. To a small Erlenmeyer flask were added 2 ml. of the tissue suspension, 0·2 ml. of a saturated As_2O_3 solution, 5 mg. of fumarate, 10 mg. of hydrazine hydrochloride and water to a volume of 4 ml. The mixture was shaken in a bath at 37° for 15 min., and then 0·5 ml. each of 10 % H_2SO_4 and 10 % Na_2WoO_4 were added. The contents were centrifuged and oxaloacetate was estimated in the filtrate. In terms of the fumarate added there was an increase of approximately 15 % in oxaloacetate. Without added fumarate or hydrazine no oxaloacetate is detectable.

According to Szent-Györgyi [Annau *et al.*, 1935] oxaloacetic acid is reduced by metabolite hydrogen to fumarate. Krebs [1936] has also suggested that oxaloacetic acid may be reduced in the oxidation of pyruvic acid. Recently Laki [Annau *et al.*, 1936] has shown by micro-chemical estimation that oxaloacetic acid added to pigeon breast muscle rapidly disappears and that there is a simultaneous increase in the amount of the fumaric-malic equilibrium mixture. That these considerations likewise apply to liver and kidney tissue is shown in the following experiment. Into Erlenmeyer flasks were placed 10 ml. of the tissue suspension, 50 mg. oxaloacetic acid and water to 21 ml. volume. The flasks were shaken in a bath at 37° for periods of 0, 2, 5, 10 and 15 min. and at the end of the periods 2 ml. each of 10 % H_2SO_4 and 10 % Na_2WoO_4 were added. The mixture was centrifuged and oxaloacetic acid, pyruvic acid, acetone, and malic acids were estimated in the clear fluid. Figs. 3 and 4 show representative experiments of this type. It is seen that as the added oxaloacetate disappears there is a simultaneous increase in the malic acid sufficient to account for about 75 % of the oxaloacetic acid lost. Since fumarate and malate exist in tissue in an equilibrium mixture, the increase in the latter upon the addition of oxaloacetic acid does not necessarily imply that it is the primary reduction product of oxaloacetate. There is likewise a slight increase in the pyruvic acid content. This may come from oxidation of a triose as suggested by Szent-Györgyi [Annau *et al.*, 1936], or since the amount of oxaloacetic acid originally added was rather large it may be the result of decarboxylation of part of it. Acetone formation, as determined with the nitroprusside test, was very slight in the liver tissue during the time of these experiments and was absent in the kidney tissue.

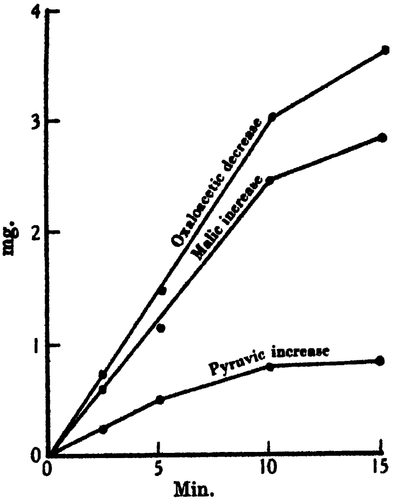

Fig. 3.

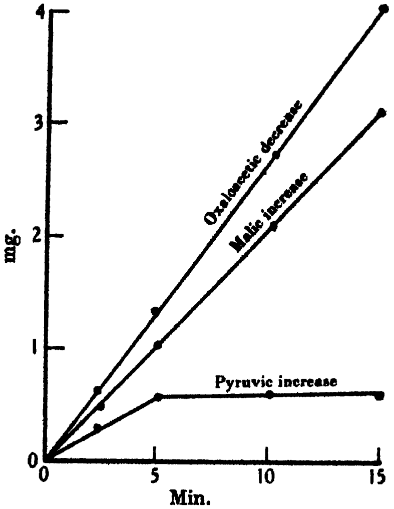

Fig. 4.

Fig. 3. Liver tissue. The curves show the decrease in added oxaloacetic acid and the simultaneous increase in malic acid together with a slight increase in pyruvic acid.

Fig. 4. Kidney tissue. The curves show the decrease in added oxaloacetic acid and the simultaneous increase in malic acid together with a slight increase in pyruvic acid.

SUMMARY.

Manometric and chemical studies on the effect of fumarate on the respiration of liver and kidney tissue were made. Fumarate increases the oxygen uptake of these tissues by conserving the initial rate of oxygen uptake. Malonate inhibits the normal respiration. With an adequate quantity of fumarate, respiration proceeds normally even in the presence of malonate. By quantitative chemical estimations it has been shown that fumarate added to liver or kidney tissue is oxidized to oxaloacetate. Likewise oxaloacetic acid added to these tissues is reduced, as indicated by the rapid disappearance of added oxaloacetic acid and the simultaneous appearance of a nearly equivalent amount of the fumaric-malic equilibrium mixture.

These facts suggest that the theory of fumaric catalysis of respiration, developed by Szent-Györgyi *et al.* for muscle tissue, applies also to liver and kidney tissue.

The writer wishes to thank Prof. Szent-Györgyi for his most generous hospitality during his stay in Szeged, and also the Josiah Macy Jr. Foundation, New York, for providing the laboratory expenses of this investigation.

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CCCXVI. THE EFFECT OF OESTROGENIC HORMONES ON LACTATION AND ON THE PHOSPHATASE[1] OF THE BLOOD AND MILK OF THE LACTATING COW.

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The numerous papers which have appeared on the endocrine control of lactation since the review by Turner [1932] have been admirably reviewed, and the present position summarized, by Nelson [1936]. It now seems clear that mammary development is promoted by the oestrogenic hormones of the ovary and the corpus luteum hormone (though the respective roles of the two types of hormone appear to differ among different species), while the possibility remains that the hypophysis may exercise some direct influence on the development of the mammary gland. The initiation and maintenance of lactation in developed mammary glands is almost certainly due to the action of the lactation-producing hormone of the anterior pituitary, usually called prolactin, which was discovered by Stricker & Grüter [1928].

In order to explain why the beginning of lactation coincides with parturition, Nelson [1932; 1933; 1934; 1935; see also Nelson & Smelser, 1933] supposes that the ovarian hormones which are known to be present in relatively high concentration in the blood during pregnancy inhibit lactation, (*a*) by inhibiting the production of prolactin by the anterior lobe of the pituitary, and possibly (*b*) by a direct inhibitory influence on the mammary gland. With the fall in the level of the ovarian hormones in the blood which occurs at parturition, the inhibitory influences are removed and milk secretion begins.

The experiments of Parkes & Bellerby [1926–27], who showed that the growth rates of litters of lactating mice receiving injections of ovarian extracts were smaller than those of controls, are in harmony with the theory of Nelson. Similar results with mice were reported by de Jongh [1933] and Robson [1934–35], though Hain [1935], working with rats, attributed the diminished growth rate to an adverse effect on maternal behaviour rather than to a physiological inhibition of lactation. Smith & Smith [1933] for the rabbit and Nelson [1934] for the guinea-pig also obtained evidence that oestrogenic hormone administration inhibits milk secretion. Results pointing in the same direction are those of Copeland [1929], who from a statistical examination of a large number of bovine milk records concluded that a slight decrease in milk yield occurs during oestrus, and those of Staffe [1930] who found conversely that the milk yields of ovariectomized cows exceeded that of controls.

The above experiments on oestrogenic hormone administration suffer from the defect that the small experimental animals used in them are not well adapted to quantitative studies on milk secretion, since with such animals the effect of the experimental treatment can only be indirectly inferred from observations on the well-being of the young.

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[1] The word "phosphatase" is used throughout this paper to denote the phosphomonoesterase A_1 [see Folley & Kay, 1936, 1].

The present work had two main objects, one of which was to study quantitatively the effect of oestrogenic hormone administration on lactation. For this work the cow was chosen as experimental animal on account of its suitability for accurate lactation studies, notwithstanding the fact that the high cost of both animals and hormones precluded the use of more than a few animals. It was hoped that by this means reliable information would be obtained on the extent to which oestrogenic hormones inhibit lactation if at all.

The second object of this work was to obtain some information on the effect of oestrogenic hormones on milk composition as part of a general programme of work now in progress at this Institute, which aims at elucidating the role of endocrine factors in controlling the chemical composition of milk (for work on thyroxine see Folley & White [1936]).

Practically no reliable information on the effect of oestrogenic hormone administration on milk composition exists, though it is reported by de Fremery [1936] that on administering oestradiol benzoate to goats which have been brought into artificial lactation by successive treatment with oestradiol benzoate and prolactin, a change from the secretion of milk to that of colostrum takes place.

Studies of the effects of oestrus and of castration on milk composition have contributed very little to this question. Copeland [1929] found that the fat content of cow's milk was slightly increased during oestrus, while the work of Staffe [1930] indicates that, apart from the fat content, which he reports to be increased, castration has practically no effect on milk composition.

EXPERIMENTAL.

Experimental animals. Four Guernsey cows belonging to the Institute herd, comprising registered and "grading up" cattle, were available for experimentation at different times. Table I gives various data relating to the experimental animals.

Table I.

Name of cow	Duration of experiment	Wt. kg.	Age in years	No. of calf	Weeks in milk at start	Remarks
Orange 4	21. ii. 36– 14. vii. 36	465	5	3	$5\frac{6}{7}$	Aborted 34 days before term
Valven 3	21. ii. 36– 27. iv. 36	425	3	2	$6\frac{4}{7}$	Aborted 66 days before term
Venus 5	29. iv. 36– 1. x. 36	458	$4\frac{3}{4}$	3	$8\frac{2}{7}$	Normal parturition
Valven 2	29. iv. 36– 5. ix. 36	345	$3\frac{1}{2}$	2	$6\frac{3}{7}$	Normal parturition

During the experiment each cow was kept indoors and given, as far as possible, a constant "winter" ration. All cows were milked twice daily at about 7 a.m. and 4 p.m., the total yield at each milking from each cow being determined on a spring balance to 0·25 lb.

Injections. Two forms of oestrogenic hormone were used. In the first experiment, with the object of administering the highest dosage of oestrogenic hormone which was available at the time, a sample of crystalline oestrone (Organon Ltd.) was injected as a part solution and part suspension in warm castor oil supplemented by a small amount of a solution of "dihydro-follicular-hormone benzoate" in sesame oil (Progynon B oleosum forte (Schering)). This latter preparation contains a mixture of isomerides, 6·7 mg. of which are equivalent in biological activity to 5·0 mg. of oestradiol benzoate. In the later experiments dihydro-follicular-hormone benzoate was used solely, either in the form of the above-named preparation or as Provetan (Schering). The prolactin (Allen and Hanbury) used in one experiment was a highly active (tested by pigeon crop gland response) 1 % suspension of crude prolactin, prepared by the method of Lyons & Catchpole [1933], in 0·3 % cresol.

Each cow received different treatment, but in each case injections were intramuscular and preceded by a control period of at least 14 days. Details are given below.

Orange 4. A total of 485 mg. oestrone and 67 mg. dihydro-follicular-hormone benzoate was injected in 5 doses spaced over 2½ days. Simultaneously the control cow, Valven 3, received similar injections of castor oil.

Venus 5. (*a*) This cow was given 5 daily injections of 20·1 mg. dihydro-follicular-hormone benzoate, Valven 2 serving as control and receiving injections of sesame oil.

(*b*) After the lapse of a month, she was given 120·6 mg. dihydro-follicular-hormone benzoate in 4 injections over 2 days, each injection being accompanied by an injection of 20 ml. prolactin. The prolactin treatment was continued as above for a further 3 days, ending on the 4th day after the cessation of the oestrogenic hormone injections with 2 injections of 30 ml. each.

Valven 2. 15 days after serving as control for Venus 5, this cow was given 87·1 mg. dihydro-follicular hormone benzoate in 4 injections spread over 4 days.

Blood analyses. At suitable intervals during the experiments, blood samples were taken from the subcutaneous abdominal veins of the cows. This was always done at approximately 10 a.m., the sera being separated the same day and preserved overnight in the refrigerator.

Total serum Ca was determined by the method of Clark & Collip [1925] using $N/200$ permanganate and with the modification that the oxalate precipitation was given 24 hours; serum inorganic P by the Briggs [1922] method; and serum phosphatase by the procedure of King & Armstrong [1934], the incubation time being 30 min. at 37°.

Milk analyses. At each milking, in addition to the determinations of milk yield, samples were taken for the determination of milk fat content by the Gerber method and of the total dry matter by evaporation. From the data thus obtained the percentage of non-fatty solids in each sample could be calculated, the correction for fat volume [Bartlett, 1933–34] being omitted.

Determinations of the phosphatase content of samples of morning milk were made from time to time, at first by the method of Kay & Graham [1933–34] and later by the King & Armstrong [1934] method. At similar intervals in the later experiments, determinations of Ca, ash and of N distribution were made on morning milk samples. The ash content was determined gravimetrically and the Ca therein estimated by permanganate titration of the oxalate. N distribution was determined by a semimicro-method developed by Rowland [1936]. The Parnas-Wagner micro-apparatus was used for all N determinations. Total N was determined on whole milk and on a trichloroacetic acid filtrate. Caseinogen was removed by isoelectric precipitation in presence of acetic acid and sodium acetate and the N in the filtrate estimated. Globulin was precipitated from an aliquot of the caseinogen filtrate by saturation with $MgSO_4$ at neutrality and the N of the precipitate found. From the data thus obtained the percentage of the milk total N as caseinogen N, albumin N, globulin N and non-protein N could be calculated.

RESULTS.

In the case of each cow the following quantities were calculated for successive three-day periods: mean daily milk yield, mean daily production of milk fat, mean daily production of milk non-fatty solids, mean percentage of fat in milk and mean percentage of non-fatty solids in milk. The results for the 3 injected cows are plotted in Figs. 1, 2 and 3.

Effect of oestrogenic hormones on milk yield.

The curves for the control cow, Valven 3 (not shown), and the appropriate portion of Fig. 3 show that injections of the solvent oils had no effect on lactation. From Figs. 1, 2 and 3 it is apparent that the oestrogenic hormone injections were always followed by a considerable decrease in milk yield. The inhibition was in all cases temporary and was obviously correlated with a transient but considerable increase in the level of oestrogenic hormone in the blood.

The occurrence of an inhibition is confirmed and additional information as to its duration in each case provided by the behaviour of the milk phosphatase content. Fig. 4 shows that in each experiment oestrogenic hormone treatment was accompanied by a tremendous increase in the milk phosphatase concentration, quickly followed by a fall to more or less normal values. It may be noted that whereas the change in milk yield in these experiments was never more than approximately 20 %, the increase in the milk phosphatase concentration varied from 300 to 1200 %.

The studies of Folley & Kay [1936, 2] on normal lactation and of Folley & White [1936] on lactation in experimental hyperthyroidism have shown that in general an increase in the level of milk secretion is accompanied by a decrease in milk phosphatase concentration and *vice versa*. In the light of these findings the present results must be taken as additional proof that oestrogenic hormones, when injected in sufficient amount, can cause an inhibition of lactation and that this inhibition only lasts as long as a high concentration of oestrogenic hormone is maintained in the blood.

An attempt was made to decide the question whether the inhibition was exerted primarily on the anterior pituitary or whether it was a direct effect on the mammary gland by giving Venus 5 a series of simultaneous injections of dihydro-follicular-hormone benzoate and prolactin. It was considered that if Nelson's theory were true, prolactin administration should partially or wholly nullify the effect of oestrogenic hormone. The result of this single experiment, in which simultaneous treatment with prolactin had no action whatever on the results of oestrogenic hormone administration (see Fig. 2) is unfortunately inconclusive. It does not support Nelson's theory but, on the other hand, it does not provide evidence against it since the prolactin dosage may have been suboptimum. Unfortunately no facilities were available at the time for doing further experiments on this point.

Effect of oestrogenic hormones on milk composition.

In the cases of all 3 cows it is evident from Figs. 1, 2 and 3 that the injections of oestrogenic hormones caused an immediate and striking increase in the non-fatty solids content of the milk. In the 2 experiments (Orange 4 and Venus 5 (*b*)) in which the total dosages were largest and spread over comparatively short periods of time, the level of non-fatty solids in the milk remained considerably increased above normal for very prolonged periods,

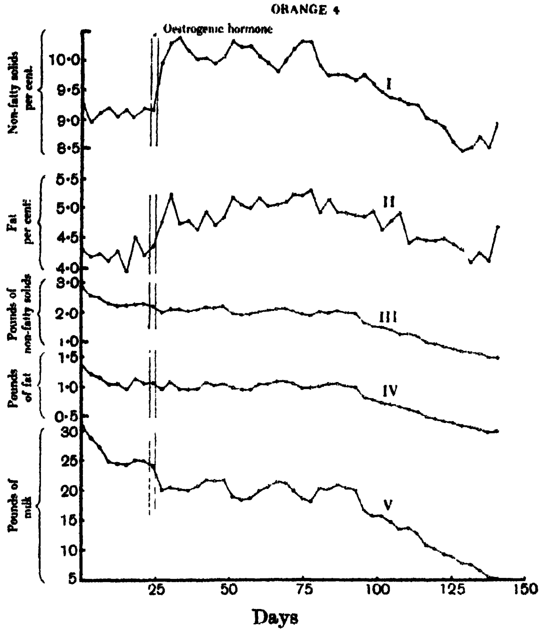

Fig. 1. I, percentage of non-fatty solids in milk; II, percentage of fat in milk; III, daily production of milk non-fatty solids; IV, daily production of milk fat; V, daily milk yield.

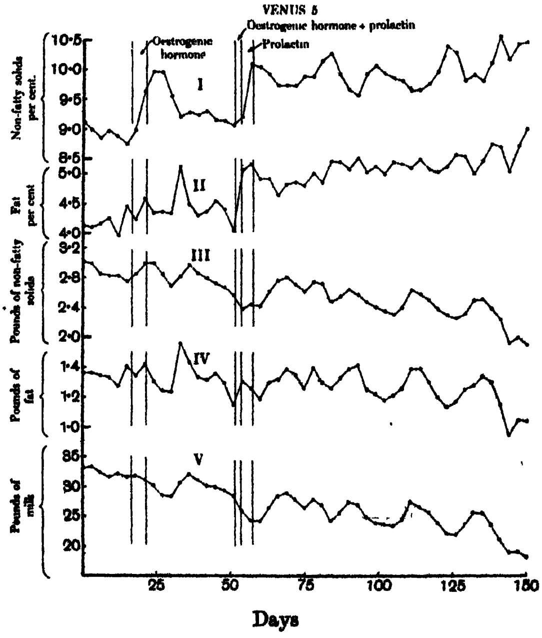

Fig. 2. I, percentage of non-fatty solids in milk; II, percentage of fat in milk; III, daily production of milk non-fatty solids; IV, daily production of milk fat; V, daily milk yield.

amounting to over 2 months in the case of Orange 4 and over 3 months in the case of Venus 5. In the 2 experiments where the dosages were smaller, and more over spread over longer periods, the effect though striking at first, quickly decreased, but even here a small effect was evident for a long time (see Fig. 3, curve 1).

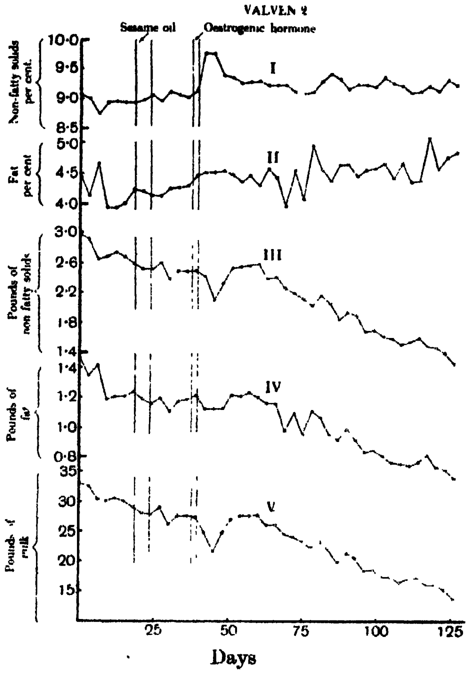

Fig. 3. I, percentage of non-fatty solids in milk; II, percentage of fat in milk; III, daily production of milk non-fatty solids; IV, daily production of milk fat; V, daily milk yield.

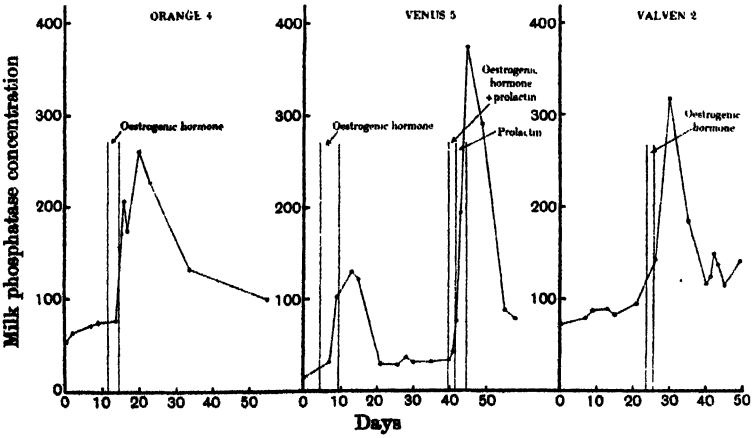

Fig. 4. In the case of Orange 4 the ordinates represent Kay and Graham [1933–34] phosphatase units in 1 ml. milk, while in the case of Venus 5 and Valven 2 they represent King and Armstrong [1934] phosphatase units in 100 ml. milk.

Similarly, the curves in Figs. 1, 2 and 3 show clearly that the experimental treatment resulted in a considerable and prolonged increase in milk fat content.

In these experiments, therefore, administration of oestrogenic hormone caused, in addition to a temporary inhibition of milk secretion in the sense of a lessened milk volume, a very prolonged increase in concentration of solids in the milk secreted. The latter finding is consistent with the possibility that oestrogenic hormones may regulate the passage into the milk of the "isotonic diluent", the existence of which was suggested by Davies [1933] as a result of a study of the composition of cow's milk poor in non-fatty solids.

Further there is some indication in the results plotted in Figs. 1, 2 and 3 that oestrogenic hormone treatment causes an actual increase in the efficiency of the mammary gland cells as regards their ability to synthesize milk constituents. Thus curves III and IV in Fig. 1 show that in the case of Orange 4 the lactation inhibition was unaccompanied by any temporary decrease in the daily production of milk fat and milk non-fatty solids. Moreover the same curves distinctly suggest that for more than 2 months after the cessation of injections the normal decline in the daily output of fatty and non-fatty solids in the milk, a decline which normally should follow approximately the same course as the decline in milk yield, was not in evidence. Both of these facts indicate that oestrogenic hormone treatment had conferred upon the mammary gland of this cow the power to synthesize milk constituents at a higher rate than before the treatment. In the cases of the other 2 cows, whilst there was a decrease in the daily output of both types of milk solids during the period of lactation inhibition, thereafter there is some indication (curves III and IV, Figs. 2 and 3) that the rate of decline in each case was slightly lessened. Unfortunately, circumstances did not permit the use for this work of two well-matched groups of cows which would have enabled this question to be decided by the application of statistical methods.

As regards the mechanism of the prolonged effects just discussed, it was considered possible that whilst the anterior lobe of the pituitary might well be concerned in the lactation inhibition, the alterations in milk composition might be due to a direct effect of oestrogenic hormones on the mammary gland, perhaps connected with the power of these substances to promote mammary development. It was, however, not possible to separate the two phenomena in the one experiment in which dihydro-follicular-hormone benzoate and prolactin were injected simultaneously. Since neither hypophysectomized nor ovariectomized cows were used, it is conceivable that the above phenomena were due to an effect of oestrogenic hormones on the anterior pituitary, which may have in turn reacted upon the ovary. In this connexion the work of Fevold *et al.* [1936], who found that administration of oestrogenic hormones increases the secretion of the luteinizing hormone by the anterior pituitary, comes to mind.

In general, these results help to explain certain previously known phenomena. The effect of pregnancy in hastening the normal decline in bovine lactation has been studied by Gaines & Davidson [1925–26] who postulated the intervention of a then unknown depressant factor. Further, Bartlett [1933–34] has demonstrated that pregnancy causes an increase in the non-fatty solids content of cow's milk. On the basis of the present results, these phenomena are probably due to the gradual rise in the level of ovarian hormone in the blood which is characteristic of gestation.

Lack of effect of oestrogenic hormones on the relative proportions of the nitrogenous constituents of milk. Fig. 5 shows the effect of injections of dihydrofollicular hormone benzoate on the density and on the total N, caseinogen N, ash and Ca contents of the milk of the two cows Venus 5 and Valven 2. The experimental treatment caused a considerable increase in the milk total N, partly due, as Fig. 5 shows, to an increase in the caseinogen N. As is to be expected, the latter phenomenon was associated with a rise in the Ca and ash contents of the milk.

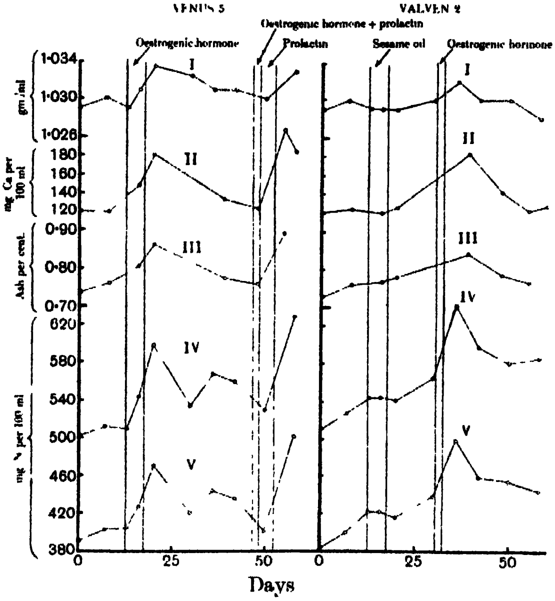

Fig. 5. I, density of milk at 20°; II, calcium content of milk; III, ash content of milk; IV, total nitrogen of milk; V, caseinogen nitrogen of milk.

De Fremery [1936], working with goats which had been brought into artificial lactation, claimed that treatment with oestradiol benzoate caused a change from the secretion of true milk to that of colostrum. From the work of Engel & Schlag [1924–25] and of Crowther & Raistrick [1916] it is established that colostrum is richer than milk in all nitrogenous fractions, perhaps the most noteworthy feature of colostrum being its very high content of globulin. Further, Engel & Schlag have found that during the gradual transition from colostrum to milk there is a progressive fall in the dry matter and ash contents of the fluid accompanied by an increase in lactose content.

Certain of the present findings, such as the increase in density and in the total solids, total N, caseinogen and ash contents of the milk following oestrogenic hormone administration, at first sight appear to be in agreement with the claims of de Fremery. The results of determinations of the N partition in the milk of two of the cows, given in Tables II and III, show conclusively, however, that such is not the case. These results show clearly that the partition of N in the milk of the experimental cows was not appreciably affected by injections of dihydro-follicular-hormone benzoate, all nitrogenous fractions participating to proportionately equal extents in the rise in total N. Had the treatment caused a transition from milk to colostrum, it is evident from the work of Crowther & Raistrick that considerable alterations in N partition would have occurred. In particular the proportion of the total N as globulin N would have increased tremendously.

Table II. *Determinations of the N partition in the milk of Venus 5.*

	Before injections		After 1st series of injections					After 2nd series of injections	
Date (1936) ...	12. v.	18. v.	21. v.	25. v.	4. vi.	10. vi.	16. vi.	24. vi.	2. vii.
mg. N per 100 ml.	512	509	545	599	534	569	561	530	630
% of total N as									
Protein N	94·6	95·1	94·9	95·1	95·3	95·3	95·0	95·2	95·5
Caseinogen N	78·5	79·2	78·2	78·7	78·5	78·0	77·8	75·8	79·8
Albumin N	10·5	11·9	12·5	11·6	9·0	11·1	10·0	11·5	10·2
Globulin N	5·6	4·0	5·0	4·9	7·8	6·1	7·2	7·9	5·6
Non-protein N	5·4	4·8	5·1	4·9	4·7	4·7	5·1	4·8	4·6

Table III. *Determinations of the N partition in the milk of Valven 2.*

	Before injections				After injections			
Date (1936) ...	18. v.	21. v.	25. v.	4. vi.	10. vi.	16. vi.	24. vi.	2. vii.
mg. N per 100 ml.	544	544	540	566	642	597	580	586
% of total N as								
Protein N	95·1	95·4	95·3	95·7	96·0	95·4	95·9	95·3
Caseinogen N	77·6	77·6	76·8	77·5	77·5	77·0	78·3	76·0
Albumin N	12·9	12·8	12·9	10·8	12·1	10·4	10·0	13·3
Globulin N	4·6	5·1	5·6	7·4	6·3	8·1	7·6	6·1
Non-protein N	4·9	4·6	4·7	4·3	4·0	4·6	4·1	4·7

The slight rise in the proportion of total N as globulin N which was first observed on 4. vi. 36 can be explained as due to the development of a mild streptococcal infection of the udder, the existence of which in both cows was inferred from bacteriological examination of the milks carried out soon after. The milk of both cows was found to be free from streptococci before the experiment started.

The effect of oestrogenic hormones on Ca and P metabolism.

In Fig. 6 are plotted for all 3 experimental cows the results of determinations of serum Ca, serum inorganic P and serum phosphatase. In all experiments the oestrogenic hormone injections caused a temporary fall in the serum total Ca.

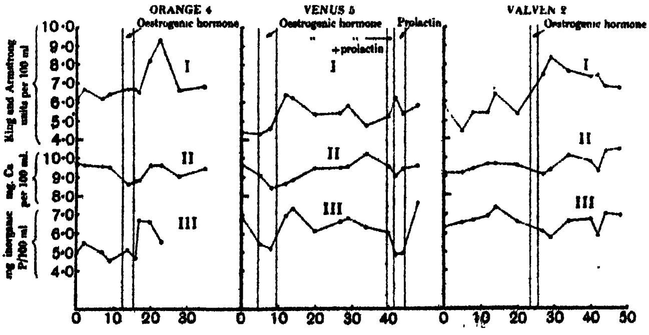

Fig. 6. I, blood serum phosphatase; II, total blood serum calcium; III, blood serum P as inorganic phosphate.

The most noteworthy feature of these results is the marked temporary rise in serum phosphatase which followed closely upon the drop in serum Ca. The results for inorganic P were more variable, but in most cases the injections appeared to cause a transient rise in this quantity.

These results for serum Ca are in general agreement with previous work. Leites [1924] found that castration of bitches caused a slow but marked rise in serum Ca while a similar effect was reported by Frei & Emmerson [1930] to follow castration of cows. The latter workers also observed a temporary fall in serum Ca following injections of oestrogenic hormones into cows.

The rise of serum phosphatase observed in these experiments is unequivocal, and taken in conjunction with the changes in serum Ca, may indicate, in the light of what is known of serum phosphatase in bone disorders [see Folley & Kay, 1936, 1], that oestrogenic hormones are capable of exercising, either directly or indirectly, far-reaching effects on the skeletal system.

SUMMARY.

1. Administration of oestrogenic hormones to lactating cows has been found to cause a considerable inhibition of lactation in the sense of a lessened milk yield. The inhibition was temporary and appeared to depend on the attainment of a high level of oestrogenic hormone in the blood.

2. After treatment with oestrogenic hormones the milks of the cows so treated contained considerably higher percentages of fat and non-fatty solids than before treatment. With high doses this effect lasted for 2–3 months.

3. The N partition in the case of such "concentrated milk" was found to be normal showing that the experimental treatment had not caused the secretion of colostrum.

4. Oestrogenic hormone administration caused a temporary fall in serum Ca quickly followed by an equally transient though considerable increase in the serum phosphatase. This was taken to indicate that oestrogenic hormones are capable of exercising generalized effects on the skeletal system.

It is a pleasure to acknowledge the continued interest of Prof. H. D. Kay in this work. I am greatly indebted to Capt. J. Golding for facilities for the determination of milk fat and total solids, to Mr J. Mackintosh for access to and management of the cows and to Dr A. T. R. Mattick for bacteriological tests on milk samples. My best thanks are due to Dr H. Neumann of Messrs Schering Ltd. for very generous supplies of dihydro-follicular-hormone benzoate, Dr A. N. Macbeth of Messrs Organon Laboratories Ltd. for a gift of crystalline oestrone and Dr N. Evers of Messrs Allen and Hanbury Ltd. for the provision of prolactin. The kindness of Dr A. S. Parkes and Dr I. W. Rowlands in testing the potency of the latter preparation is gratefully acknowledged. I also desire to thank the Government Grant Committee of the Royal Society for a grant towards the cost of the apparatus used in this work, as well as the Milk Marketing Board, a grant from which contributed towards the expenses.

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CCCXVII. THE EFFECT OF LIGHT ON THE VITAMIN C OF MILK.[1]

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(*Received 26 October 1936.*)

In estimating vitamin C chemically in milk by the method of Birch *et al.* [1933], Kon [1933] observed very marked fluctuations in the concentration of that vitamin from day to day.

The possible causes of this phenomenon were investigated by Mattick & Kon [1933], who ultimately found that milk which originally gave a positive vitamin C titration failed to reduce the indophenol reagent after a short exposure to light in glass bottles. Some time later Booth & Kon [1934] showed that 90 % of the original power of reducing the reagent could be restored after short exposures to light by treating the milk with hydrogen sulphide. Longer exposure entailed greater irreversible losses.

An example of the effect of light will be found in Table I.

Table I. *Vitamin C estimations carried out on milk from a pint bottle before and after half-an-hour's exposure to sunlight.*

Ascorbic acid in mg./100 ml. of milk.

Before exposure		After exposure		Control wrapped in black paper and exposed	
Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S
1·95	2·38	0·00	1·41	1·95	2·38

The titrations were carried out as described on p. 2275. The higher titration given by the unexposed milk after treatment with H_2S is most probably due to previous exposure to light in the dairy (see p. 2278).

The present paper deals with detailed investigations carried out since the preliminary papers were published.

EXPERIMENTAL METHODS.

Choice of analytical methods.

Booth & Kon [1934], when reducing the reversibly oxidized substance, treated the milk exposed to light with H_2S; the milk was then deproteinized with trichloroacetic acid and the serum freed from hydrogen sulphide and titrated.

When the method was applied to milk in which vitamin C had been destroyed by heating and aeration in the presence of copper a small residual titration was obtained in every case. As it was highly unlikely that any vitamin C could have survived the drastic treatment, estimations were also carried out by the mercuric acetate method of Emmerie & van Eekelen [1934]. The results are set out in Table II. In all cases 0·1 ml. of a 3 % solution of $CuSO_4$ was added to 100 ml. of milk. The milk was then heated to 90° and air was bubbled through for various lengths of time. It will be seen from the table that the method of Emmerie and van Eekelen indicates complete destruction.

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[1] Read in part before the Biochemical Society, June 12th, 1936 [Watson & Kon, 1936].

Table II. *The destruction of vitamin C in milk by aeration at 90° in the presence of copper measured by the method of Booth & Kon [1934] and of Emmerie & van Eekelen [1934].*

			Vitamin C content of milk in mg. per 100 ml.			
			Original milk		Heated milk	
No. of exp.	Time of heating hr.	Method of titration	Before H_2S treatment	After H_2S treatment	Before H_2S treatment	After H_2S treatment
1	0·5	B. & K.	2·25	2·86	0·00	0·49
2	2·0	,,	2·05	2·63	0·00	0·45
3	2·0	,,	1·87	2·41	0·00	0·45
4	2·75	,,	1·60	2·60	0·00	0·40
5	2·75	,,	4·60*	5·50*	0·00*	0·44*
6	2·75	E. & v. E.	1·95	2·25	0·00	0·00

* About 3 mg. of ascorbic acid were added to this milk before heating.

On the other hand a residual titration (equivalent to some 0·4–0·5 mg. of ascorbic acid per 100 ml. of milk) is obtained in all cases in which the method of Booth & Kon [1934] is used. The residual titration is very constant and is independent of the time of heating and of the amount of ascorbic acid originally present in milk.

When the two methods are applied to normal milk the Emmerie & van Eekelen technique gives lower values and the difference is exactly the same as the residual titration obtained above (Table III). This makes it almost certain that this residual titration is due to substances other than ascorbic acid.

Table III. *Comparison of the mercuric acetate method (M.A.) with the trichloroacetic acid method involving reduction followed by deproteinization (T.A.R.) and with the trichloroacetic acid method involving deproteinization followed by reduction (T.A.D.).*

	mg. of ascorbic acid per 100 ml. of milk after reduction with H_2S				Difference, mg./100 ml.			
Method	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
M.A.	1·96	2·10	2·42	2·24	0·41	0·47	0·49	0·44
T.A.R.	2·37	2·57	2·91	2·68				
T.A.D.	—	2·10	2·42	2·24	—	—	—	—

In the mercuric acetate (Emmerie & van Eekelen) method the H_2S treatment is applied not to milk but to serum after deproteinization.

When the trichloroacetic acid method used by Booth & Kon [1934] was so altered that deproteinization preceded reduction, results identical with those given by the mercuric acetate method were obtained. The residual titration is therefore no doubt due to the action of H_2S on milk with the formation of a substance which reacts with the indophenol reagent.

Ascorbic acid added to milk is recovered by the mercuric acetate method and by the modified trichloroacetic acid method quantitatively and with equal accuracy (Table IV). The error is well within the limits of accuracy of the methods.

Table IV. *Recovery of ascorbic acid added to milk.*

5·7 mg. of ascorbic acid added to 100 ml. of milk. Results in mg. ascorbic acid/100 ml. of milk.

Method	Ascorbic acid content of milk		Ascorbic acid		% error
	Original milk	Milk and ascorbic acid	Added	Recovered	
M.A.	2·24	7·83	5·7	5·6	-1·7
T.A.D.	2·24	7·83	5·7	5·6	-1·7

The following procedure was finally adopted for the estimation of ascorbic acid in milk.

Method and technique of estimation of vitamin C in milk.

In a later part of this paper (p. 2284) evidence is offered that on exposure of milk to light ascorbic acid undergoes a reversible oxidative change to a biologically active product, very probably dehydroascorbic acid. This can be again reduced by H_2S and reacts then with the indophenol reagent. It is further shown that the substance (or substances) which does not react with the reagent after reduction with H_2S is biologically inert. For the sake of simplicity the knowledge of these findings will be assumed from the beginning of the paper.

The titration before H_2S treatment is referred to as "reduced ascorbic acid"; that obtained after it, measuring the reduced and reversibly oxidized forms of vitamin C, is described as "total ascorbic acid". All values are expressed as mg. of ascorbic acid in 100 ml. of milk.

Indicator. An approximately 0·04 % solution of 2:6-dichlorophenolindophenol in pre-boiled, glass-distilled, water. The indicator is standardized against lemon juice, which in turn is titrated with 0·01 *N* iodine according to the method of Bessey & King [1933].

Preparation of milk. To 25 ml. of milk 15 ml. of 20 % trichloroacetic acid are added. The mixture is allowed to stand for a few minutes after shaking and is then filtered.

Estimation of reduced ascorbic acid. 5 ml. of clear filtrate are titrated from a microburette with the standardized indophenol reagent until a faint pink colour, which persists for 30 sec., is obtained.

Estimation of total ascorbic acid (*reduced and reversibly oxidized*). Through the remainder of the filtrate a moderate stream of H_2S is passed for about 10 min. The container is then stoppered and left overnight in the dark. On the following morning the serum, saturated with H_2S, which has become cloudy on standing, is filtered and the H_2S is removed by passing a rapid stream of oxygen-free nitrogen until the emergent gas fails to stain moist lead acetate paper. This takes on the average about half an hour. Titration is then carried out as above.

The nitrogen is purified as follows. Commercial nitrogen from a cylinder is passed through 4 wash-bottles containing alkaline pyrogallol and through a sulphuric acid tower followed by a trap. It then passes into an electrically heated combustion tube filled with copper turnings. The emerging nitrogen is led through a wash-bottle containing alkaline glucose and methylene blue[1] into the milk serum.

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[1] A 1 % solution of methylene blue is added drop by drop to a hot 1 % solution of glucose in *N*/10 NaOH until a faint blue is obtained. The temperature is then raised until the solution becomes colourless.

INVESTIGATION OF THE EFFECT OF LIGHT ON THE ASCORBIC ACID OF MILK.

The work reported in the preliminary papers [Kon, 1933; Mattick & Kon, 1933; Booth & Kon, 1934] was carried out on milk in commercial pint bottles. In the present investigation it was found more convenient to expose milk in smaller quantities, usually 25 ml. For this purpose the milk was placed in 3 oz. sample bottles shaped like milk bottles, with walls 4 mm. thick. To speed up the reaction the milk was frequently exposed to direct sunshine. The milk was obtained either from individual Shorthorn cows or from pooled Shorthorn milk produced on the Institute's farm.

Effect of exposure to different sources of light and to different wave-lengths.

Exposure to artificial light. 25 ml. of milk were exposed for 2 hours at a distance of 1 ft. to the light of a 12 volt 48 watt motor-car head lamp bulb (Siemens, gas-filled) working at 10 V. and 2·8 A. The milk gave an original titration of 1·33 mg./100 ml. of reduced and 1·71 mg./100 ml. of total ascorbic acid and a titration, after exposure, of 0·37 and 0·59 mg./100 ml. respectively. A control sample protected from the light remained unchanged.

Exposure to the light of a mercury vapour lamp in glass and quartz. 25 ml. quantities of milk were exposed as above in glass and quartz for 0·25, 0·5 and 1 hour at a distance of 25 cm. to the unscreened radiations of a Kelvin, Bottomley and Baird mercury vapour lamp working at 106 V. and 2·5 amp. D.C. The results are given in Table V.

Table V. *Effect of exposure in glass and quartz to the light of a mercury vapour lamp on the vitamin C content of milk.*

R. reduced, T. total ascorbic acid in mg./100 ml.

			Time of exposure					
	Original milk		15 min.		30 min.		60 min.	
Material	R.	T.	R.	T.	R.	T.	R.	T.
Glass	2·32	2·64	1·28	1·76	1·12	1·60	0·32	1·04
Quartz			1·04	1·36	0·80	1·44	0·24	1·04

The figures show that a greater reversible change and greater destruction are brought about by exposure in quartz, especially in the case of shorter exposures. The difference is, roughly, some 20 %. According to Coblentz *et al.* [1926] some 30 % of the radiations of a mercury vapour lamp up to 600 mμ (the following paragraphs show that light beyond that wave-length is inactive) are of a wave-length shorter than 290 mμ. As ordinary glass cuts off at about 310 mμ, the loss due to passing through glass would be more than 30 %. Shorter ultraviolet rays do not therefore appear to be any more potent than the longer radiations given off by the mercury vapour lamp. According to Ellis & Wells [1925], the visible radiations of the mercury vapour lamp are chiefly concentrated in two yellow-green lines at 576·4 and 579·1 mμ and a green line at 546·1 mμ. In view of the experiments with a sodium vapour lamp reported below, it is doubtful whether the first two lines play any role in the reaction. The active rays which pass through glass may therefore be due to the green line, and/or to the strong lines in the near ultraviolet (at 365–365·4, 398·4 and 404·6–407·8 mμ).

Exposure to daylight through transparent cellophanes of different colours. The relative destructive effects of different wave-lengths have been studied by exposing

25 ml. samples of milk in boiling-tubes of 25 mm. internal diameter covered with the following colours of cellophane (300 grade)—red, brown, lemon, blue and heliotrope. In addition milk in a clear glass tube and in a completely darkened tube was also exposed. Absorption spectra of most of the cellophane wrappings used in this investigation will be found in a paper by Davies [1934]. The samples were exposed for 1 hour to February daylight. The results are given in Fig. 1. It will be seen that red cellophane protected the vitamin C almost completely, whilst the greatest change was found in the tubes covered with blue or violet (heliotrope) cellophane.

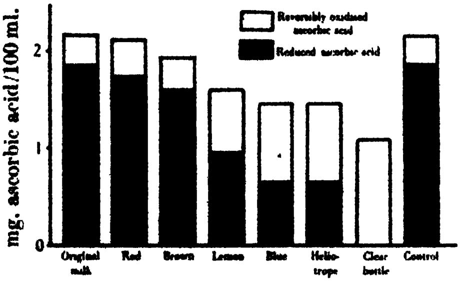

Fig. 1. The ascorbic acid content of samples of milk after 1 hour's exposure to daylight passing through cellophane wrappers of different colours.

Exposure to the light of a sodium lamp. The relative inefficiency of the longer wave-lengths in the destruction of vitamin C in milk, demonstrated by the previous experiment, has been further confirmed by exposing milk to the monochromatic radiations of a sodium vapour lamp (Osira, G.E.C.) working at 1·35 amp. and 14·4 V. Two 25 ml. samples were exposed, one for 1 hour at a distance of 1 ft., whilst the second was similarly exposed for 1 hour, and then for another hour at half the distance. The second sample received therefore the equivalent of 5 hours' exposure at a distance of 1 ft. As Table VI shows, the sodium vapour light was totally without effect on the ascorbic acid of milk.

Table VI. *Effect of monochromatic sodium vapour light on the ascorbic acid of milk.*

R. reduced, T. total ascorbic acid in mg./100 ml.

Original milk		Exposed milk					
		A. Exposure for 1 hr. at 1 ft.		B. Exposure as A + 1 hr. at 6 in. = 5A.		Control exposed in black wrappers	
R.	T.	R.	T.	T.	T.	R.	T.
1·79	2·27	1·79	2·24	1·79	2·24	1·79	2·27

It will be remembered that the car lamp bulb (p. 2276) working at 28 watts for 2 hours at a distance of 1 ft., caused the destruction of some 65 % of the ascorbic acid originally present in milk. Compared with this the lack of effect of the sodium lamp working at 19 watts for the equivalent of 5 hours at 1 ft. shows that light of wave-length 589 mμ (and, doubtless, of longer wave-length) is inactive. The slight destruction caused by light filtered through "brown" or "lemon" cellophane is no doubt due to admixture of shorter wave-lengths. The experiments described above show that the shorter visible rays and the near ultraviolet are chiefly concerned with the vitamin C reaction. In the case of daylight acting through glass the relatively much greater intensities available in the blue and violet regions of the spectrum would make them chiefly responsible for the light effect.

Penetration of the active rays of sunlight through milk. A thin layer of milk transmits an appreciable amount of light. The following experiment was carried out in order to see whether the transmitted rays are active. Milk was exposed to June sunlight in rectangular cells made of cleaned photographic plates placed one behind the other in a black box which was open at one end. The layer of milk in the cells was 7 mm. thick (Table VII).

Table VII. *Penetration of the active rays of June sunlight through milk.*

Time of exposure min.	Reduced ascorbic acid in mg./100 ml.			
	Original milk	1st cell	2nd cell	3rd cell
0	A. 2·06	—	—	—
	B. 2·26	—	—	—
15	—	A. 0·00	A. 1·99	—
30	—	A. 0·00	A. 1·92	—
60	—	A. 0·00	A. 1·62	—
	—	B. 0·00	B. 1·41	B. 2·23

Sufficient light passes through a 7 mm. thickness of milk to cause an appreciable drop in reduced ascorbic acid in the second cell. A layer of 14 mm., however, prevented action taking place in the course of an hour's exposure. It is noteworthy that, according to Leikola [1932], milk absorbs more strongly in the red than in the blue.

Effect of oxygen.

When the dissolved oxygen of milk is completely replaced by an inert gas the reaction does not take place. A container with milk was evacuated three times on a high vacuum oil pump and each time the vacuum was released in an atmosphere of oxygen-free nitrogen. The nitrogen was then bubbled through the milk for half-an-hour. The container was then stoppered and the milk exposed for 2 hours to afternoon February sunshine. The results are presented in Fig. 2.

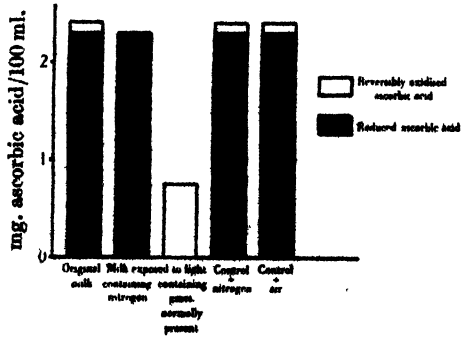

Fig. 2. Effect of light on the ascorbic acid of milk in the presence of oxygen or of an inert gas. 2 hours' exposure to afternoon February sunshine.

Effect of previous heating of the milk.

Ascorbic acid is reversibly oxidized under the action of light just as readily in milk which had been pasteurized (30 min. at 63·5°) as in raw milk. For example, a 15 min. exposure to June sunshine brought the concentration of reduced ascorbic acid in a sample of laboratory pasteurized milk from 2·10 mg./100 ml. to nil. The total ascorbic acid fell at the same time from 2·17 to 1·72 mg./100 ml. These results are very similar to data obtained under similar conditions for raw milk (Fig. 5 and p. 2280).

It would seem unlikely that the enzymes of milk, which are largely inactivated during pasteurization, play a role in the process of oxidation.

Comparison of the effects of light on whole and separated milk.

25 ml. samples of whole milk and of milk from which most of the fat had been removed by centrifuging were exposed for 25 min. to pale noon sunshine in early March. No difference was found between the samples in either the reduced ascorbic acid (which was completely oxidized during the exposure) or the total ascorbic acid. It should be observed that the amount of fat left in separated milk is quite large compared with the amount of ascorbic acid.

Effect of length of exposure; evidence of two separate reactions.

25 ml. quantities of milk were exposed to June skyshine. At the end of 1, 2, 5 and 8 hours analyses for vitamin C were carried out (Fig. 3). It will be seen that all the ascorbic acid originally present in the reduced form had undergone reversible oxidation within the first hour of exposure, and that irreversible changes took place progressively throughout the whole length of the experiment. It is at once apparent that two separate reactions must have taken place: firstly a reversible change of the ascorbic acid to a substance no longer able to bleach the indophenol reagent unless treated with hydrogen sulphide; to effect this first change both light and oxygen are essential and this reaction does not proceed in darkness (Tables XIII, XV and XVI); secondly a more gradual change of the product of the first reaction to a substance which cannot be caused by H_2S treatment to give a positive test with indophenol and is, as will be shown later, biologically inactive. The question arises whether or not light is essential to the second reaction as well.

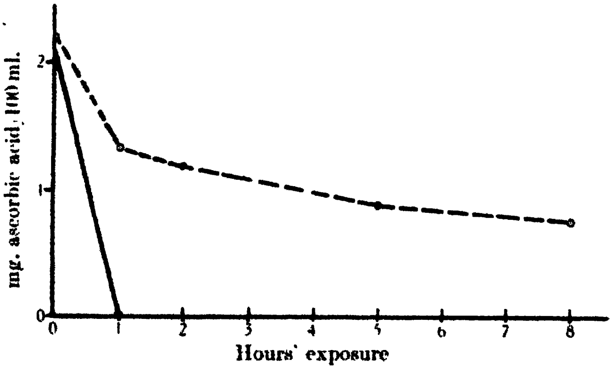

Fig. 3. Effect of length of exposure to light on the concentration of vitamin C in milk.
- - - Total ascorbic acid. ——— Reduced ascorbic acid.

Is light essential to the second reaction?

25 ml. samples of milk were exposed for half an hour to dull daylight (end of March). One sample and one control were titrated immediately after exposure and the remainder were placed in a dark cupboard and an exposed sample and a

control were removed and titrated after 1, 2, 3, 4 and 5 hours (Fig. 4). In the course of the half-hour exposure the reduced ascorbic acid has disappeared completely, leaving some 1·6 mg./100 ml. of the reversibly oxidized form. This has continued to change in the absence of light so that after a further 5 hours only 1/3, some 0·55 mg./100 ml., is left. The shape of the curve is very similar to that obtained in the course of continuous exposure to light (Fig. 3), and it is quite obvious that when only reversibly oxidized ascorbic acid is left the reaction proceeds spontaneously for a long time without the agency of light. The truth of this statement is confirmed by the experiments presented in Fig. 5. Five 25 ml. samples of milk were exposed to May sunlight for the shortest time necessary to cause the complete disappearance of the reduced form of ascorbic acid—in this case 15 min.—and 4 other samples were exposed 4 times as long. One sample of the first group was analysed immediately after exposure, another at the same time as the first sample of the second group. Thereafter samples

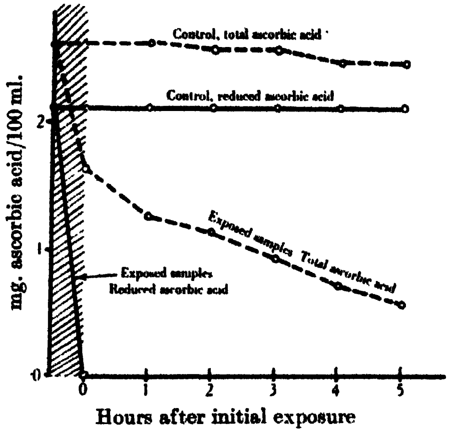

Fig. 4. Behaviour in darkness of the ascorbic acid of milk after reversible oxidation caused by exposure to light.

▨ Exposure to light, ½ hour.

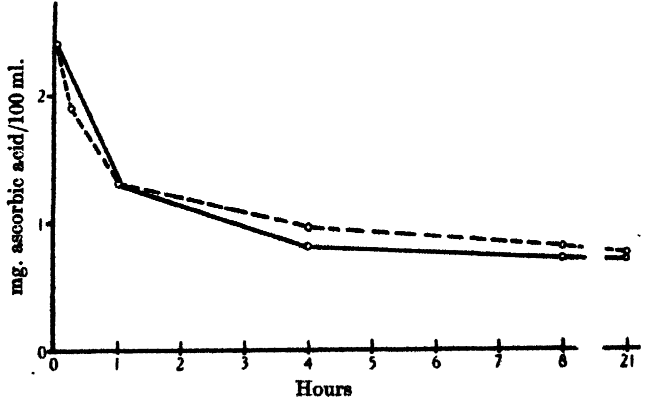

Fig. 5. Rate of decomposition of reversibly oxidized ascorbic acid after different periods of exposure to light.

——— Exposed for 1 hour. - - - Exposed for ¼ hour.

from both groups were analysed together at intervals. The graph shows that the shapes of the two curves were very similar and that almost identical end values were reached.

Investigation of the first reaction.

Order of reaction. In the experiments described so far the exposure to light had been mostly too long to allow of a measurement of the rate of formation of the reversibly oxidized from the reduced form of ascorbic acid. As a rule the reduced ascorbic acid was found to have disappeared completely in the course of the exposure. For this reason estimations at frequent intervals were carried out and the rate of oxidation was followed. It was found that measurements made every 5 min. gave enough points for the calculation of the order of the reaction. Fig. 6 presents the results of two series of observations. The logarithm of the concentration of reduced ascorbic acid (mg. per litre) is plotted against the time of exposure and the straight lines obtained show unmistakably that the reaction is a unimolecular one. The bottom line was obtained in the shade

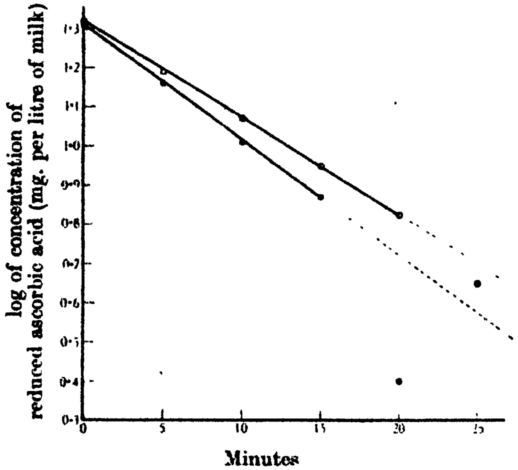

Fig. 6. Order of reaction of the reversible oxidation by light of the ascorbic acid of milk.

Table VIII. *Reversible oxidation of the ascorbic acid of milk under the action of light.*

Concentration of the reduced form at 5 min. intervals and velocity constants of reaction.

Time min.	Reading mg./100 ml. 1st series	Reading mg./100 ml. 2nd series	Difference 1st series	Difference 2nd series	Velocity constant 1st series	Velocity constant 2nd series
0	2·06	2·08				
			0·62	0·52	0·0716	0·0575
5	1·44	1·56				
			0·41	0·38	0·0670	0·0558
10	1·03	1·18				
			0·29	0·29	0·0661	0·0564
15	0·74	0·89				
			0·49	0·22	0·2171	0·0568
20	0·25	0·67				
			0·25	0·22	—	0·0796
25	0·00	0·45				
			—	0·45	—	—
30	—	0·00				

on the forenoon of a cloudless June day, the top one on a dull day similarly in June. For very low concentrations the points, as would be expected, are off the lines. The last point in the top series is well within the experimental error. The actual readings and the velocity constants calculated from the equation $k=\frac{2\cdot303}{t}\log_{10}\frac{A_0}{A}$ (where A_0 and A are the initial and final concentrations in the time interval t) are given in Table VIII.

Effect of temperature. 25 ml. quantities of milk in darkened bottles were placed (1) in a refrigerator at 0°; (2) at room temperature, 21°; (3) in an incubator at 37° until the milks had attained the temperature of the surroundings. They were then exposed for 10 min. to skyshine (July) and titrations were carried out immediately afterwards (Table IX).

Table IX. *Effect of temperature on the reversible oxidation of the reduced ascorbic acid of milk under the action of light.*

Reduced ascorbic acid in mg./100 ml.

		After 10 min. exposure to skyshine	
Original milk	0°	21°	37°
2·00	1·41	1·04	0·52
Velocity constant	0·0350	0·0654	0·1347

The temperature coefficient of the reaction, calculated according to Van't Hoff [1898] is 1·44 for the range of temperature 0–37°.

The second reaction.

Little can be said at present with regard to the order of the second reaction. Judging from the shape of the curves in Figs. 3, 4 and 5 there is little doubt that it is not unimolecular.

Fig. 5 shows clearly that the reaction does not proceed to completion. A similar conclusion will be reached after inspection of the data of Table X, in which are shown the results of titrations of samples of milk which had been exposed for 15 min. to May sunshine—just long enough to convert all reduced ascorbic acid into the reversibly oxidized form. The samples were then kept in darkness and titrated at intervals.

Table X. *Decomposition of reversibly oxidized ascorbic acid in milk kept in darkness.*

Original milk Ascorbic acid		Concentration of reversibly oxidized (total) ascorbic acid after exposure to light (mg./100 ml.)								Control after 22 hr. Ascorbic acid	
Reduced	Total	Immediately after exposure	½ hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	22 hr.	Reduced	Total
2·20	2·42	1·99	—	1·03	0·88	0·74	0·59	—	0·59	2·13	2·36
1·98	2·20	1·77	1·18	0·88	—	0·74	—	0·74	0·63	1·84	2·20

Discussion.

The observations bearing on the effect of light on the ascorbic acid of milk may be considered from two points. In the first instance they may be compared with the behaviour of ascorbic acid itself in aqueous media. Secondly, other changes which are known to take place in milk under the action of light can be considered.

As is known, ascorbic acid readily undergoes oxidation to dehydroascorbic acid which is unstable in aqueous solution and undergoes a spontaneous change by the addition of the elements of water and opening of the lactone ring to 2:3-diketo-1-gulonic acid. Dehydroascorbic acid itself can be converted quantitatively into ascorbic acid by reduction with hydrogen sulphide, but the diketogulonic acid cannot be reduced to more than a slight extent with this reagent [Herbert *et al.* 1933].

The close similarity of this chemical behaviour of ascorbic acid with the changes undergone under the action of light by the ascorbic acid of milk leaves little room for doubt that the first step of the actinic reaction consists in the formation of dehydroascorbic acid. Not only is the substance in milk obviously a product of oxidation, but it shares further with dehydroascorbic acid the properties of reverting to ascorbic acid when treated with H_2S and, as will be shown later, of being biologically active. As regards the further decomposition of the first oxidation product, it is possible, and even probable, that diketogulonic acid is spontaneously formed in milk. The evidence is only of negative character. The substance in milk, like the gulonic acid, is neither reduced by H_2S nor does it exhibit biological activity. While it is very likely that the two substances are identical it will be well to remember that a reaction in the complex environment of milk may take a very different course from that in a simple aqueous solution.

It has been known for some time (Davies [1936, 1, 2] has reviewed the literature on the subject) that changes of an oxidative character take place in milk under the action of light. The changes which occur when milk is exposed to sunlight in clear glass bottles are generally attributed to photochemical activation of the dissolved oxygen or oxygen diffusing in. Outwardly the action manifests itself in the tainting of milk which, owing to the oxidation of the fat acquires a "cardboard" flavour.

The effect of light on the vitamin C of milk is doubtless a part of the general oxidative changes induced in milk by photo-activation. Whereas the oxidation of the fat is of an autocatalytic nature, the reversible oxidation of vitamin C stops when the light is removed. It may be reasonably assumed that ascorbic acid is present in true solution and that the velocity of reaction between ascorbic acid and oxygen under the influence of light is relatively high. Ascorbic acid would thus have priority over the emulsified fat in the competition for the available oxygen. In any case, the unimolecular character of the curve of reversible oxidation of the ascorbic acid of milk indicates that a relatively large excess of oxygen is available for the purpose.

It has been shown in Table I that half an hour's exposure to sunlight is sufficient to change all the ascorbic acid in a pint bottle of milk into the reversibly oxidized form. On the other hand the experiments described on p. 2278 indicate that a layer of milk 14 mm. thick efficiently prevents any actinic action in the course of 1 hour's exposure to strong sunlight and that a layer of half that thickness cuts the activity down very considerably. The rapid spread of the reaction through 70 mm. of milk in a pint glass bottle must therefore be explained otherwise than by the penetration of the actinic rays into the milk. It is possible that a chain reaction is involved.

THE EFFECT OF LIGHT ON ASCORBIC ACID ADDED TO MILK.

So far in this paper the identity with ascorbic acid of the photo-labile substance in milk giving the indophenol reaction has been assumed, though not finally proved. The following experiments make the evidence in favour of this assumption, already very strong, virtually complete.

Synthetic ascorbic acid added to milk behaves on exposure to light in exactly the same way as the ascorbic acid originally present. Several examples of the effects of light on milk fortified by addition of ascorbic acid are shown in Table XI. A synthetic preparation (B.D.H.) was used.

Table XI. *Effect of light on synthetic ascorbic acid added to milk.*

R. reduced, T. total ascorbic acid in mg./100 ml.

No. of exp.	Original milk		mg. ascorbic acid added per 100 ml.	Milk + ascorbic acid		Titration after 1 hr. exposure		Titration after 2 hr. exposure	
	R.	T.		R.	T.	R.	T.	R	T.
1*	2·14	2·49	About 4·5	6·89	7·14	0·00	3·10	0·00	2·59
						Titration after 3 hr. exposure		Titration after 7 hr. exposure	
2†	1·93	2·30	8	10·09	10·24	0·74	5·20	0·00	1·63
3†	1·86	2·15	8	9·95	10·17	0·00	3·34	0·00	0·89
4†	1·93	2·23	8	10·09	10·25	0·00	3·86	0·00	1·71

* 25 ml. samples exposed. † 150 ml. samples exposed.

It was found that with the higher concentration of ascorbic acid longer exposures were necessary to obtain complete reversible oxidation. Exp. 2 (Table XI) shows, for example, that even after 3 hours' exposure, some ascorbic acid had been left in the reduced form. Obviously, owing to the longer exposures, variations in the intensity of light were much more likely to occur, and these account for the marked differences between separate runs. Otherwise the chemical behaviour of the added ascorbic acid leaves no reasonable doubt as to the identity with vitamin C of the substance in milk which gives the indophenol titration.

BIOLOGICAL TESTS OF THE EFFECT OF LIGHT ON THE VITAMIN C ACTIVITY OF MILK.

The results so far described do not solve the problem of the vitamin C potency of milk which has been exposed to light. For this reason biological tests have been carried out.

The planning of the biological tests presented certain difficulties. It was evident for example that milk after exposure to light would have to be fed and consumed at once to obtain a correct measure of the activity of the reversibly oxidized substance. Delay in consumption would bring about the further spontaneous changes which have been described in the preceding pages.

This implied feeding by pipette, but the relatively low concentration of ascorbic acid in milk would make it necessary to give some 50 ml. within a short time—again a serious difficulty. Finally, the work involved in exposing the milks to light and carrying out the titrations, made it desirable to shorten as far as possible the experimental period. It was decided to increase the concentration of ascorbic acid in milk, by addition of the synthetic substance and thus cut down the volume of milk required for feeding. As for the method of testing, a combination of Harris & Ray's [1932] curative technique with an estimation of the ascorbic acid content of the adrenals [Svirbely & Szent-Györgyi, 1933] and of the liver was adopted. Macroscopical lesions noticed at autopsy were also taken into consideration. Non-pregnant female guinea-pigs were used for the test as well as males. The animals, weighing initially 250–300 g., were kept for 3 weeks on the scorbutic diet of Eddy [1929] and were then used for the tests.

It was originally hoped to feed three types of milk: milk containing ascorbic acid only in the reduced form, milk with a similar content of ascorbic acid but only in the reversibly oxidized form and finally milk giving no indophenol titration even after treatment with H_2S. In practice, owing to the long exposures to light required by milk containing added ascorbic acid (discussed in the preceding chapter) it was necessary to feed one level of reduced ascorbic acid and 2 lower levels of the reversibly oxidized form.

The treatment of the milk was as follows: the milk was assumed to contain 2 mg./100 ml. of ascorbic acid (final measurements were not completed till the following day). 8 mg. of synthetic B.D.H. ascorbic acid were then added per 100 ml. of milk, giving a fivefold increase in the concentration of ascorbic acid. A part of this milk was then fed by pipette without delay to guinea-pigs. Two

Table XII. *Biological tests of the effect of light on the ascorbic acid in milk.*

Treatment	Average daily intake of ascorbic acid (mg.)	Total ascorbic acid in adrenal (average)		Total ascorbic acid in liver (average)		Scurvy score	Average gain or loss in weight during experimental period g.
		mg./g.	mg. in organ	mg./g.	mg. in organ		
First experiment. Average daily intake of milk 15 ml. per guinea-pig. Duration of experiment 7 days.							
Unexposed milk; 3♀	1·42 (reduced form)	0·22	0·050	0·05	0·71	0	+ 1
Milk exposed 3 hr.; 3♀	0·61 (reversibly oxidized)	0·13	0·029	0·04	0·58	+ (+)	− 12
Milk exposed 7 hr.; 3♀	0·30 (reversibly oxidized)	0·08	0·020	0·04	0·51	+ + (+)	− 23
Negative controls; 2♀	0·00	0·04	0·014	0·03	0·46	+ + + +	− 58
Second experiment. Average daily intake of milk 13 ml. per guinea-pig. Duration of experiment 14 days.							
Unexposed milk; 4♂	1·30 (reduced form)	0·23	0·060	0·06	0·80	Trace (?)	+ 10
Milk exposed 3 hr.; 4♂	0·52 (reversibly oxidized)	0·10	0·023	0·04	0·80	+ (+)	+ 9
Milk exposed 7 hr.; 4♂	0·24 (reversibly oxidized)	0·07	0·018	0·03	0·42	+ + (+)	− 31
Ascorbic acid 1st level; 2♂	0·50 (reduced form)	0·12	0·032	0·05	0·96	+	+ 19
Ascorbic acid 2nd level; 1♂	1·00 (reduced form)	0·28	0·084	0·05	0·82	Trace	+ 34
Negative controls; 3♂	0·00	0·03	0·009	0·03	0·29	+ + + +	−122

other samples were then exposed in clear glass bottles of 150 ml. capacity and internal diameter of 5 cm. on a window ledge facing south. The exposures lasted generally 3 and 7 hours. Examples of typical runs are given in Table XI. These milks were given after exposure to guinea-pigs in the same way as the unexposed milk. All feedings were done in a darkened room. Two separate feeding tests were carried out. In one test only female guinea-pigs were used and the milks were given for 7 days. The other test lasted a fortnight and employed only male guinea-pigs. In the second test control guinea-pigs receiving an aqueous solution of ascorbic acid were also included. The results are given in Table XII.

The results of the experiments show conclusively that exposure of milk to light entails a loss of the vitamin C activity and it appears that, while the reversibly oxidized form of ascorbic acid is biologically active, the product of the subsequent decomposition is inactive. When the results obtained in the second experiment with the milk exposed for 3 hours are compared with those given by 0·5 mg. of ascorbic acid the difference in activity between the reduced and reversibly oxidized forms does not seem to be marked. This is more in agreement with the findings of Hirst & Zilva [1933] than with those of Roe & Barnum [1936]. The method of titration of ascorbic acid in milk used in the present study appears to be a reliable guide to its vitamin C activity.

EFFECT OF EXPOSURE OF MILK TO LIGHT UNDER PRACTICAL CONDITIONS.

The following experiments were carried out in order to find out to what extent the vitamin C content of milk can be affected by exposure to light in the normal routine of handling sale milk as when e.g. it is delivered on the door-step.

Pint and half-pint bottles of sale milk from the Institute's dairy were exposed in sun or shade (end of May) for half-an-hour, 1 hour and 2 hours. They were then placed in darkness and analysed at intervals (Table XIII and Fig. 7). The degree of destruction varies materially with the intensity and the time of exposure, and to a less extent with the size of the bottle. The loss of vitamin C is quite noticeable, and an exposure for half an hour in the sun followed by half an hour in the dark will reduce to half the amount originally present in the bottle. Even after short exposure in the shade, standing for a few hours in darkness will reduce by half the vitamin C available in a bottle of milk.

RESISTANCE TO HEAT TREATMENT OF THE VITAMIN C OF MILK BEFORE AND AFTER EXPOSURE OF THE MILK TO LIGHT.

It is generally known that pasteurization diminishes the vitamin C content of milk. The loss is especially severe in the presence of copper from the pasteurizing plant or dairy utensils, which acts as a catalyst. But even in the absence of copper a varying proportion of the vitamin, depending on the method of pasteurization, is destroyed. As the ascorbic acid of milk, reversibly oxidized under the action of light, is in general less stable than the reduced form of the vitamin, it was of interest to investigate the behaviour of these two substances in the course of pasteurization.

Half-gallon amounts of milk were obtained from the dairy shortly after milking. They were placed in aluminium vessels and pasteurized in the laboratory by the holder method (30 min. at 145–150° F.). Samples were analysed before and after pasteurization. The heat treatment and sampling were carried out in the absence of light (Table XIV).

Table XIII. *Effect on the vitamin C content of sale milk of exposure in pint and half-pint bottles in sun and shade for varying lengths of time.*

R. reduced, T. total ascorbic acid in mg./100 ml.

No. of exp.	Length of exposure hr.	Type of exposure	Type of bottle	Original milk R.	Original milk T.	Immediately after exposure R.	Immediately after exposure T.	1 hr. after exposure R.	1 hr. after exposure T.	3 hr. after exposure R.	3 hr. after exposure T.	6 hr. after exposure R.	6 hr. after exposure T.	24 hr. after exposure R.	24 hr. after exposure T.	Control after 24 hr. R.	Control after 24 hr. T.
I	0·5	Sun	1 pt.	1·93	2·38	0·00	1·41	0·00	1·11	0·00	0·97	0·00	0·82	0·00	0·59	1·78	2·23
		Shade				1·11	1·93	1·11	1·78	1·11	1·63	1·11	1·48	0·97	1·19		
	1·0	Sun				0·00	1·04	0·00	0·74	0·00	0·74	0·00	0·59	0·00	0·59		
		Shade				0·85	1·71	0·82	1·56	0·82	1·45	0·82	1·34	0·82	1·11		
	2·0	Shade				0·69	1·41	0·69	1·26	0·69	1·26	0·69	1·11	0·45	0·74		
2	0·5	Shade	½ pt.	1·82	2·30	0·82	1·80	0·59	1·26	0·59	1·08	0·59	1·04	0·52	0·97	1·63	2·15
			1 pt.			1·19	1·86	0·97	1·63	0·97	1·41	0·97	1·19	0·52	0·97	1·71	2·23
	1·0	Shade	½ pt.			0·59	1·63	0·48	1·19	0·56	1·08	0·56	0·97	0·37	0·97	1·63	2·15
			1 pt.			0·67	1·63	0·56	1·26	0·59	1·26	0·59	0·97	0·45	0·74	1·71	2·23

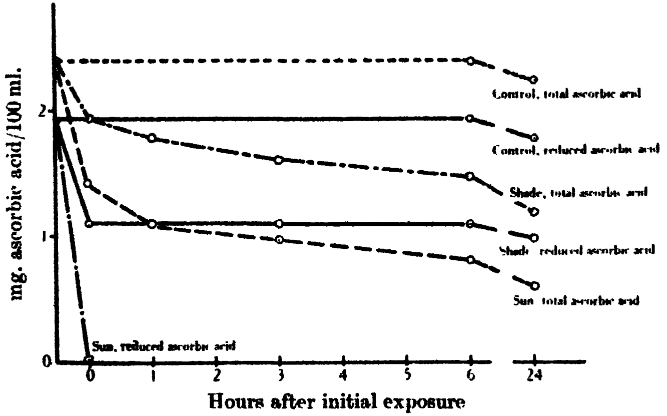

Fig. 7. Effect of half an hour's exposure to light in sun and shade on the vitamin C content of sale milk in pint bottles.

Table XIV. *Effect of pasteurization on the reduced and reversibly oxidized ascorbic acid of milk.*

R. reduced, T. total, R.O. reversibly oxidized ascorbic acid in mg./100 ml.

No. of exp.	Raw milk		Pasteurized milk		% loss in pasteurization		Raw milk	Ascorbic acid destroyed in pasteurization
	R.	T.	R.	T.	R.	T.	R.O.	
1	1·72	2·10	1·57	1·72	8·7	18·1	0·38	0·38
2	1·95	2·25	1·87	1·95	4·1	13·3	0·30	0·30
3	1·87	2·10	1·65	1·65	11·8	21·4	0·23	0·45
4	1·78	2·04	1·78	1·78	0·0	12·7	0·26	0·26
5	1·65	2·10	1·57	1·83	4·8	12·9	0·45	0·27
6	1·80	2·25	1·72	1·80	4·4	20·0	0·45	0·45
7	1·87	2·02	1·76	1·80	5·9	10·9	0·15	0·22
8	1·78	2·08	1·78	1·78	0·0	14·4	0·30	0·30
Average	1·80	2·12	1·71	1·79	4·9	15·5	0·31	0·33

The table shows that the loss caused by pasteurization in the total (reduced and reversibly oxidized) ascorbic acid originally present in milk is more than 3 times greater than that suffered by the reduced form. This means that it is chiefly the reversibly oxidized acid that has been destroyed. In fact it can be seen from the last columns of Table XIV that the reversibly oxidized acid present in raw milk accounts quantitatively for the net loss due to pasteurization (that is for the difference in total ascorbic acid before and after the heat treatment).

It has been found that milk taken directly from the udders of normal cows contains only the reduced form of ascorbic acid. These findings will be presented in a separate paper but the effect of heat treatment on the ascorbic acid of such milk is given here. 2½ pints of milk were obtained in the absence of light directly from the udder through the teat-cup lining of a milking machine into an aluminium pasteurizing vessel. The milk was pasteurized immediately at 145–150° F. for half-an-hour. Samples were taken in darkness for analysis before and after pasteurization. The results obtained with the milks of 2 cows are given in Table XV. These results show conclusively that reduced ascorbic acid is not affected by pasteurization provided that catalysing metals are absent.

Table XV. *The effect of pasteurization in the presence or absence of added copper on the vitamin C content of milk obtained in darkness.*

R. reduced, T. total ascorbic acid in mg./100 ml.

No. of exp.	Raw milk		Pasteurized milk, no copper added		Pasteurized milk, 7 parts per million of copper added as $CuSO_4$	
	R.	T.	R.	T.	R.	T.
1	2·17	2·17	2·10	2·17	—	—
2	2·02	2·02	2·02	2·02	0·00	0·45

For a final experiment 4 pint bottles of freshly produced milk were obtained from the dairy. 2 were kept in dark wrappers and 2 were exposed to intermittent July sunshine for 35 min., which was the minimum time necessary to produce almost complete reversible oxidation. The two types of milk were then pasteurized for half an hour in aluminium vessels. Table XVI confirms again the instability of the reversibly oxidized and the resistance to heat treatment of the reduced form of ascorbic acid.

Table XVI. *Effect of pasteurization before and after exposure to light on the ascorbic acid content of milk.*

R. reduced, T. total, R.O. reversibly oxidized ascorbic acid in mg./100 ml.

Raw milk		Pasteurized milk		Raw milk	Ascorbic acid destroyed in pasteurization
R.	T.	R.	T.	R.O.	
		Milk not exposed to light.			
2·15	2·37	2·15	2·11	0·22	0·26
		Milk exposed to light.			
0·26	1·63	0·22	0·52	1·37	1·11

It will be noticed that the figure for total ascorbic acid in unexposed pasteurized milk is lower than that for the reduced. The difference is however within the experimental error of the method.

All these experiments make it clear that it is primarily the amount of reversibly oxidized ascorbic acid present in milk that governs the loss of vitamin C during pasteurization by the holder method and not necessarily the presence or absence of oxygen during the process. By analogy with the mechanism of the change of pure ascorbic acid in aqueous solutions:

$$\text{Ascorbic acid} \xrightarrow{+O_2} \text{dehydroascorbic acid} \xrightarrow{+H_2O} \text{2:3-diketo-}l\text{-gulonic acid},$$

the thermal decomposition of the reversibly oxidized ascorbic acid in milk may well not require the presence of oxygen. This view is certainly in agreement with the experiments of Rundberg [1934], who found no difference between pasteurization at 63° for half an hour *in vacuo* or in contact with air, as far as the destruction of vitamin C was concerned.

Differences in the initial exposure of milk to light and therefore in the amount of reversibly oxidized ascorbic acid present in the milk probably account for the very divergent statements found in the literature as to the extent to which vitamin C is destroyed by pasteurization.

SUMMARY.

1. Milk giving a positive chemical test for vitamin C fails to reduce the indophenol reagent after exposure to daylight through glass; the reducing power can be restored to a varying extent by treatment with H_2S, but irreversible losses always take place.

2. Visible light of short wave-length (blue and violet) is mainly responsible for the reaction. Ultraviolet light is also probably active. Yellow and red are almost without effect.

3. The action of light does not take place in the absence of oxygen. The reversible oxidation under the action of light follows the laws of a unimolecular reaction and has a temperature coefficient of 1·4 for the range of temperature 0–37°. It is possibly a chain reaction. The decomposition of the reversibly oxidized ascorbic acid is of a more complicated order and does not run to completion.

4. It is suggested that the effect of light on the ascorbic acid of milk is a part of the general oxidative changes induced in milk by the actinic activation of oxygen. It is also suggested that the mechanism of the breakdown of ascorbic acid in milk is similar to the known oxidative decomposition of this substance, namely that dehydroascorbic acid is formed in the reversible oxida-

tion and that the opening of the lactone ring takes place in the course of the further irreversible changes.

5. Synthetic ascorbic acid added to milk behaves, under the action of light, in the same way as the ascorbic acid originally present.

6. Tests on guinea-pigs carried out on milks to which ascorbic acid had been added confirm the chemical findings. They show namely that the reversibly oxidized substance formed under the action of light is biologically active, but that the decomposition products which fail to decolorize the indophenol reagent after the treatment with H_2S are devoid of activity.

7. A pint bottle of milk exposed under practical conditions on the doorstep for half-an-hour in the sun and then kept for 1 hour in the darkness loses fully half of its original antiscorbutic properties.

8. Pasteurization by the holder method destroys the reversibly oxidized, but does not affect the reduced, form of ascorbic acid in milk. Milk as secreted by the normal cow contains only reduced ascorbic acid. The amount of destruction of vitamin C caused by pasteurization in the absence of catalytic metals depends on the previous exposure of the milk to light.

Our best thanks are due to Miss D. V. Dearden for much help in obtaining the samples of milk.

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CCCXVIII. ON THE AMOUNT OF ASCORBIC ACID IN BLOOD AND URINE. THE DAILY HUMAN REQUIREMENTS FOR ASCORBIC ACID.

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THE investigations described in this contribution were undertaken [van Eekelen, 1936] to answer the following questions: (1) Do the ascorbic acid content of the blood and the daily urinary output of it depend on the amount of vitamin C stored in the human organism? Is it possible to get an insight into the state of saturation of a given subject, for the special purpose of detecting hypovitaminosis, by determining the ascorbic acid in blood or in urine? (2) What are the normal daily requirements for ascorbic acid of human adults?

Van Eekelen *et al.* [1933, 1] and Johnson & Zilva [1934] have shown that, following intake of large doses of ascorbic acid, the organism can be saturated to such an extent that a further dose of ascorbic acid is partly or entirely excreted in the urine. In this way the storage of ascorbic acid in a certain subject can be investigated by determining the amount of ascorbic acid required to reach the point of saturation. We shall consider this point to be reached when a daily dose of 250–400 mg. ascorbic acid results in a definite rise of urinary output (about 30 mg. above the normal average). This does not indicate complete saturation, in which case nearly the total amount taken in is excreted.

The questions arise: What is the amount of ascorbic acid in the blood when this point of saturation has been reached? Do the amount in the blood and the daily urinary output of ascorbic acid decrease when a saturated experimental person lives for some time on a diet devoid of vitamin C? When a decrease results an increase can be expected by saturating the person again.

By this experiment it is possible also to calculate the daily requirements for the vitamin supposing that the quantity of ascorbic acid necessary to saturate the subject at the end of the dietetic period equals the amount required during this period. No reasons against this supposition could be detected. To be sure that the dietetic prescriptions were followed as strictly as possible and that the urinary examinations could be done in time the writer himself was the experimental subject.

METHODS.

Determination of ascorbic acid in urine. The ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol standardized against crystalline ascorbic acid (about 10 ml. indicator corresponds to 1 mg. ascorbic acid). Urine contains besides ascorbic acid other substances reducing the indicator, e.g. ergothioneine [Sullivan & Hess, 1933] and thiosulphates [van Eekelen, 1934]. For this reason it is necessary to remove these interfering substances by precipitation with mercuric acetate [Emmerie, 1934; Emmerie & van Eekelen, 1934; van Eekelen & Emmerie, 1936]. The urine is examined immediately after it has been voided.

Determination of ascorbic acid in blood. In blood also reducing substances other than ascorbic acid are present (e.g. ergothioneine, glutathione) which can be removed likewise by precipitation with mercuric acetate. Thus, the blood being precipitated with trichloroacetic acid, mercuric acetate is added, the mixture neutralized with $CaCO_3$ until slightly acid (Congo red paper remaining unaltered, blue litmus paper turning to red) and treated with H_2S [Emmerie & van Eekelen, 1934].

Hardly any reducing substances are present when the blood filtrate is titrated after precipitation with trichloroacetic acid (before the treatment with mercuric acetate and H_2S) even when pure ascorbic acid has been added previously. From this experiment we have earlier concluded [van Eekelen *et al.* 1933, 2] that the ascorbic acid in the blood is present in the reversibly oxidized state and that an added quantity of ascorbic acid will be reversibly oxidized by the blood. The following determinations however demonstrate in agreement with Kellie & Zilva [1935; 1936] that this supposition is not true [van Eekelen, 1935].

Table I. *mg. ascorbic acid per 10 ml. ox blood, plasma and erythrocytes.*

	After precipitation with CCl_3COOH	After reduction with H_2S	After precipitation with CCl_3COOH and $Hg(CH_3COO)_2$ and reduction with H_2S
Blood	0	0·28	0·042
Plasma	0·035	—	0·034
Erythrocytes	0	—	0·050
Blood + 0·9 mg. ascorbic acid	0·15	—	0·93
Blood, centrifuged; in 5 ml. plasma	0·75	—	0·75
Blood + 4·92 mg. ascorbic acid	3·31	—	4·91*
Plasma + 4·92 mg. ascorbic acid	4·89	—	4·91†

* Calculated 4·95. † Calculated 4·94.

Table I shows: (1) Ascorbic acid is present both in the plasma and in the erythrocytes. (2) Precipitation with mercuric acetate is necessary because after reduction with H_2S without precipitation with mercuric acetate a larger amount of reducing substances is found. This demonstrates the presence of reducing substances other than ascorbic acid. (3) In plasma ascorbic acid is present in the reduced state. (4) During the precipitation with trichloroacetic acid, the ascorbic acid becomes reversibly oxidized if erythrocytes are present. This oxidation occurs especially when the blood is oxygenated. After treatment with CO_2 or CO only a small part of the ascorbic acid is oxidized (Table II).

Table II.

	After precipitation with CCl_3COOH	After precipitation with CCl_3COOH and $Hg(CH_3COO)_2$ and reduction with H_2S
10 ml. aerated blood + 4·92 mg. ascorbic acid	3·31	4·91
10 ml. blood saturated with CO_2 + 4·92 mg. ascorbic acid	4·72	4·89
10 ml. blood saturated with CO + 4·92 mg. ascorbic acid	4·72	4·89

Thus we have offered sufficient evidence that the determination of ascorbic acid in blood is unreliable without treatment of the trichloroacetic extracts with mercuric acetate and H_2S. From the data above results the possibility of determining the vitamin in serum directly, without treatment with mercuric acetate and H_2S [Gabbe, 1934; Farmer & Abt, 1935; Abt *et al.*, 1936; Taylor *et al.*, 1936]. However, it is advisable to apply the mercuric acetate and H_2S procedure because in serum also other interfering reducing substances may occur or the ascorbic acid may be partly reversibly oxidized (e.g. after haemolysis).

Experimental details. The experimental person was a man, aged 30 years, height 178 cm., weight 90 kg. The investigations were done from August to December 1935. The diet devoid of vitamin C contained bread, butter, cheese, eggs and water. For the saturation tests crystalline ascorbic acid (Hoffmann-La Roche) was taken.

The course of the experiment is given in Table III and Fig. 1.

Table III.

Date (1935)	mg. ascorbic acid taken in	ml. urine excreted	Daily urinary output mg. ascorbic acid	mg. ascorbic acid per l. blood	g. faeces excreted per day	mg. ascorbic acid in faeces	Number of petechiae. Left arm (*L*), right arm (*R*)
29. vii	—	—	—	—	—	—	—
30	—	742	13·1	—	—	—	—
31	—	875	12·4	—	—	—	—
1. viii	—	833	13·4	11·2	175	4·6	—
2	250	929	16·6	11·7	—	—	—
3	250	780	23	16·8	120	3·6	—
4	—	793	17·2	15	140	3·8	—
5	250	769	*45·6*	17	143	4·3	—
6	—	675	15·8	15	140	4·1	—
7	—	754	13·7	—	76	3·2	—
8	—	—	—	—	128	3·3	—
13	—	—	—	9·6	—	—	—
17	—	1046	10·5	—	—	—	—
18	—	903	12	—	—	—	—
19	—	770	10·2	—	—	—	—
21	—	—	—	6	—	—	—
22	—	847	11·9	—		—	—
23	—	819	11·2	—	—	—	—
28	—	902	10·2	—	—	—	—
29	—	830	10·1	—	—		—
30	—	—	—	4·3	—	—	—
4. ix	—	—	—	—	—	—	*L* 0
7	—	910	9·2	—	—	—	—
8	—	845	8·4	—	143	4·2	—
9	—	—	—	3·7	—	—	—
13	—	855	9·1	—	—	—	—
14	—	777	8	—	—	—	*L* 0
15	—	790	7·6	—	—	—	—
16	—	—	—	3·1	—	—	—
23	—	713	9	2·5	—	—	—
24	—	807	8·1	—	—	—	—
28	—	889	8	—	—	—	*L* 2, *R* 1
29	—	—	—	—	142	4·7	—
1. x	—	—	—	3	—	—	—
4	—	727	6·7	—	—	—	—
9	—	769	7·8	—	—	—	—
11	—	—	—	2·3	—	—	—
12	—	—	—	—	—	—	*L* 3, *R* 2
21	—	660	7·2	2·2	—	—	—
26	—	—	—	—	—	—	*R* 1
28	—	789	7	1·9	145	7·4	—
29	2 × 250	678	8·8	—	—	—	—
30	2 × 250	764	9·1	—	—	—	—
31	2 × 250	699	10·9	5	—	—	—

Table III (*cont.*).

Date (1935)	mg. ascorbic acid taken in	ml. urine excreted	Daily urinary output mg. ascorbic acid	mg. ascorbic acid per l. blood	g. faeces excreted per day	mg. ascorbic acid in faeces	Number of petechiae. Left arm (*L*), right arm (*R*)
1. xi	2 × 250	688	15·1	—	186	8·7	—
2	250	682	15·9	8·9	—	—	—
3	250	688	12·9	—	—	—	—
4	—	678	12·2	9·4	—	—	—
5	250	656	14·5	—	—	—	—
6	250	727	19	—	—	—	—
7	250	646	*35·5*	13·3	—	—	—
8	—	594	15	14·4	—	—	—
9	—	761	14·4	—	—	—	—
11	—	556	12·9	—	—	—	—
12	—	—	—	10·6	—	—	—
16	—	614	10·5	8·3	—	—	—
21	—	—	—	6·6	—	—	—
26	—	625	10·1	—	—	—	—
27	—	607	9·2	5·1	—	—	—
28	250	661	9·5	—	—	—	—
29	250	728	10·3	—	—	—	—
30	250	651	10·7	—	—	—	—
1. xii	250	683	13·3	—	—	—	—
2	250	737	15·8	—	—	—	—
3	250	604	21·6	—	—	—	—
4	250	556	*39·5*	13·2	—	—	—

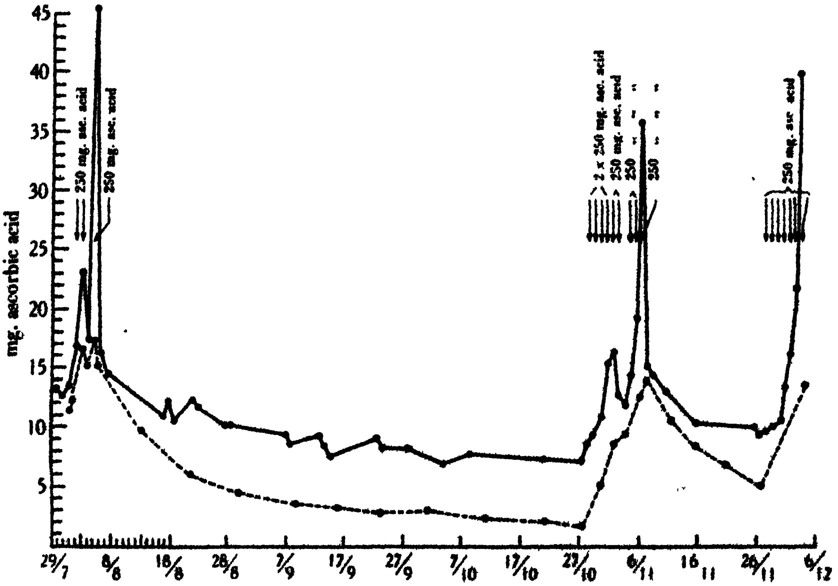

Fig. 1. •—• mg. ascorbic acid, daily urinary output.
•– – –• mg. ascorbic acid per l. blood.

From this table it will be seen that the diet deprived of vitamin C began on 29 July. On the next 3 days the amounts of ascorbic acid excreted in the urine were determined (about 13 mg. per day). After an intake of 250 mg. daily during 3 days a definite rise in the urinary output was observed (a surplus excretion of about 30 mg. on 5 August).

By determining the ascorbic acid content of the faeces it was shown that an increased excretion with the faeces did not occur when large doses of ascorbic acid were given (see Table III).

Following this saturation the diet devoid of vitamin C was continued until 29 October. At intervals the ascorbic acid content of the blood and the daily urinary output were determined. The amount in the blood being 11·2 mg./l. before the experiment began was 11·7 mg./l. after 250 mg. had been taken and increased to 16·8 mg. after the second dose of 250 mg. (determination 3 hours after intake of ascorbic acid). On the day of this increased amount in the blood a somewhat larger urinary excretion was observed (23 mg. instead of 13 mg.). The next day (4 August) no ascorbic acid was taken in; the amount in the blood decreased to 15 mg./l. On 5 August 250 mg. were taken in again and a greatly increased excretion (45·6 mg.) resulted. Compared with the amount on 3 August the content of the blood only rose to 17 mg./l. (3·5 hours after intake). This increase was transitory, the next day 15 mg. were found. So it appeared that a surplus excretion of ascorbic acid in the urine occurs when a certain level in the blood (about 15 mg./l.) is surpassed. In the course of the dietetic period the daily urinary output slowly decreases: after 83 days from about 13 mg. to about 7 mg. with, however, large fluctuations. Compared with this observation the decrease of the amount in the blood is more important however only during the first period of the experiment (after 25 days from 15 mg. to 4·3 mg./l.). During the next period the decrease was less marked (after 59 days from 4·3 to 1·9 mg./l.).

After 84 days of the regimen ascorbic acid was again taken. To reach the point of saturation more quickly two doses of 250 mg. were taken daily during the first days, then daily doses of 250 mg. were taken (as at the beginning of the experiment) to avoid the point of saturation being suddenly largely exceeded. 3250 mg. were necessary to restore saturation (7 November, 35·5 mg. were excreted in urine). In the course of the saturation the amount in the blood and in the urine increased. At the point of saturation the amount in the blood was 14·4 mg./l. The regimen was continued for another 20 days; the amount in the blood decreased to 5·1, the urinary output to 9–10 mg. per day. By taking 1750 mg. of ascorbic acid saturation was again reached. The amount in the blood rose to 13·2 mg.

From these experiments it follows that together with the development of a vitamin C deficiency the amounts of ascorbic acid in the blood and in the urine decrease. This agrees with the findings of Abt *et al.* [1936] and Taylor *et al.* [1936] on the ascorbic acid content of blood serum. It appears to be possible to get an insight in the state of saturation of a given subject by determining the ascorbic acid content of the blood or of the urine. For this purpose the estimation in the blood is preferable, for it is less complicated because it needs only one examination whilst the excretion in the urine has to be determined during 24 hours and immediately after voiding. Furthermore there are rather large fluctuations in the daily output of the same subject. Moreover, the daily output is very unequal in different subjects with about the same state of saturation, in the nearly saturated state varying from 9 to 27 mg. The amount in the blood decreases more and more quickly than that of the urine. The relation between the state of saturation and the content in the blood however is not linear.[1] For this reason three classifications only appear reasonable: about or above 13 mg./l. signifies saturation, below 4 mg. has to be considered as insufficient, between 13 and 4 mg. more or less sufficient.

In illustration of the facts described above some determinations are given of the ascorbic acid content of the blood before and after saturation with ascorbic acid of three patients probably suffering from scurvy.

In two patients the initial ascorbic acid contents were 3·8 and 2·2 mg./l. After saturation with daily oral doses of 300 and 400 mg. to an amount of 3300 and 4000 mg. ascorbic acid the concentrations in the blood increased to 12·8 and 13 mg./l. In the third case the patient was saturated with 400 mg. ascorbic acid daily given *per os* or intravenously. The amount in the blood was estimated just before the daily dose of ascorbic acid was given, except on 25 February (5 hours after administration). The ascorbic acid content of the urine was investigated during 5 hours after administration of the ascorbic acid. The results are given in Fig. 2.

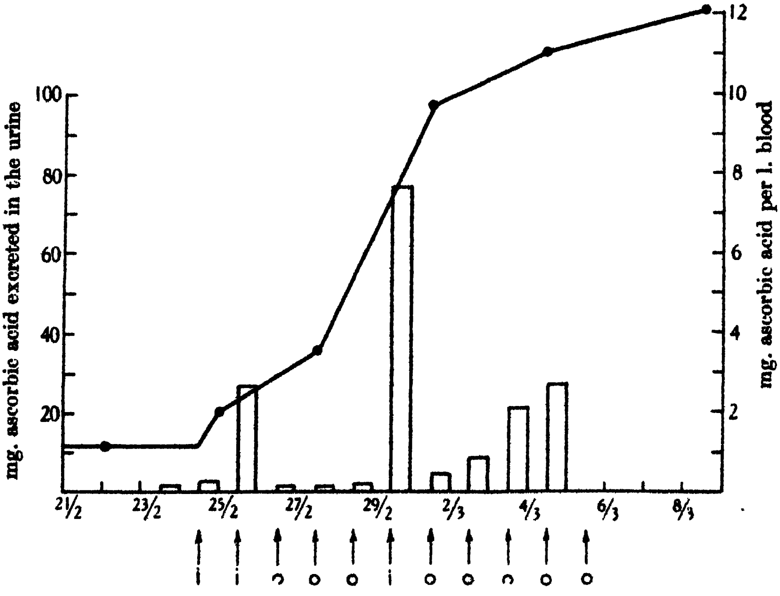

Fig. 2. i = Intravenous. o = per os.

The figure shows that after intravenous administration of ascorbic acid an increased urinary excretion already results before the point of saturation is reached. This can be explained by the supposition that after the injection the amount of ascorbic acid in the blood rises for a short period above the excretion level.

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[1] *Note added 4 December 1936.* Determining the concentration of ascorbic acid in the blood and the quantity necessary to saturate different subjects I have found that the relation between these values is linear. A concentration in the blood of 4 mg. per l. corresponds with a saturation dose of about 2 g.; 8 mg. per l. corresponds with a dose of 1 g. ascorbic acid (for subjects with a body weight of 70 kg. The decrease of ascorbic acid in the blood during a dietetic period (see Fig. 1) is not linear because the use of ascorbic acid decreases during this period, as has been demonstrated in this paper.

The capillary resistance test.

According to Göthlin [1931] and Dalldorf [1933] the capillary resistance depends on the state of saturation; a lowered capillary resistance should indicate a deficiency.

During the first experiment described above some changes in the capillary resistance might have been expected at the end of the dietetic period. A considerable deficiency could be demonstrated by the saturation test (viz. 3250 mg. ascorbic acid had to be taken to reach the point of saturation). Yet the capillary resistance remained normal (see Table III). The technique of the measurements was that given by Göthlin (50 mm. Hg pressure during 15 min.). It appears noteworthy that at the end of the experiment (after 84 days) no other symptoms of vitamin C deficiency than fatigue and irritability could be observed; the body-weight remained constant.

Daily human requirements.

During the first experiment lasting from 6 August to 7 November, 3250 mg. ascorbic acid were taken (Table III). To calculate the amount stored or used in the body from the quantity taken must be subtracted the amount of ascorbic acid excreted above the normal average during the last few days of intake (about 30 mg.). The experiment lasting 94 days the daily requirements amount to

$$\frac{3220}{94} = 34 \text{ mg.}$$

This result differed somewhat from that of a similar informative experiment done in 1934. In this experiment the ascorbic acid content of the urine was estimated directly, without treatment with mercuric acetate and H_2S. This omission did not influence the main result of the experiment because the aim was the registration of a definite peak in the urinary output of ascorbic acid. That experiment lasted 40 days, 1850 mg. were required, the daily requirements amounted to 45 mg.

The difference between these two experiments can possibly be accounted for by their different durations. The decreased urinary output of ascorbic acid at the end of the dietetic period eventually indicates restricted requirements. The requirements should be dependent on the amount of the vitamin stored in the organism. To test this supposition another experiment was done lasting only 27 days, from 7 November to 4 December (Table III, Fig. 1). During this period the organism has not been depleted appreciably (the amount in the blood became 5·1 mg./l.). 1750 mg. minus 38 mg. (surplus excreted) were required to saturate the organism again. From this determination a daily requirement of 63 mg. can be calculated (1712/27). Thus we may suppose an adaptability of the daily requirement to the amount of ascorbic acid stored in the organism. The requirement decreases when the organism becomes depleted. During a period without strongly depleted depots the amount used daily is 63 mg.

According to this method [van Eekelen, 1936] van Wersch [1936, 1, 2] has determined the daily requirements for himself (man 38 years, height 168 cm., weight 72 kg., duration of the experiment 31 days) and for a female patient suffering from degeneration of the skeleton probably caused by chronic scurvy (age 18 years, height 136 cm., weight 53 kg., duration of the experiment 17 days). Requirements amounting to 56 and 44 mg. respectively resulted (the concentrations in the blood after saturation were respectively 13 and 13·7 mg./l.). Evaluating the daily requirements of these three experimental persons per kg. body weight, a close agreement can be observed when the weights are corrected to the body lengths (calculated from Shall's [1933] height-weight-age table). (See Table IV).

Table IV.

	Height cm.	Weight kg.	Corrected weight	Daily require-ments mg. ascorbic acid	Daily require-ments per kg.	Daily requirements per kg. corrected weight
Van Eekelen	178	90	74·6	63	0·70	0·84
Van Wersch	168	72	67	56	0·78	0·84
Patient J	136	53	?	44	0·83	0·83

From this the conclusion may be drawn that corpulent people require per kg. body weight less ascorbic acid than slender ones. Possibly the adipose tissues require little ascorbic acid.

From the data presented it follows that the daily requirements for healthy adults weighing 70 kg. amount to about 60 mg.

For a further discussion of the daily human requirements I will refer to the communication of Heinemann in this number of the *Journal* (p. 2299).

SUMMARY.

The content of ascorbic acid in blood and its urinary output depend on the amount of vitamin C taken in and on the quantity stored in the organism. Saturation of the organism coincides with a certain level in the blood (about 13 mg./l.). This level being surpassed surplus excretion in the urine results. To estimate the rate of saturation the determination of ascorbic acid in the blood has proved to be the most reliable method (besides the saturation test). A method to determine daily human requirements for ascorbic acid is described.

The daily requirements are dependent upon the amount stored in the organism, they are largest when the subject does not become markedly unsaturated.

The daily dose required for adults weighing 70 kg. amounts to about 60 mg. under normal conditions.

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CCCXIX. I. ON THE RELATION BETWEEN DIET AND URINARY OUTPUT OF THIOSULPHATE (AND ASCORBIC ACID). II. HUMAN REQUIREMENTS FOR VITAMIN C.

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PART I.

FOR several vitamins the quantity necessary to maintain health depends on the composition of the diet. Rats, for instance, need more vitamin D while on a diet with little phosphorus and with abundant calcium than with P and Ca offered in equal doses. It is known also that man requires more vitamin B_1 the greater the carbohydrate content of the food.

As to vitamin C, Ahmad [1936] and Chakraborty & Roy [1936] have recently shown that the reducing capacity of urine (against dichlorophenolindophenol) increases during a high protein diet. If there really exists a greater urinary excretion of ascorbic acid during a high protein diet, greater requirements of the organism, too, may be expected. Both authors titrated the urine without previous treatment: they did not eliminate interfering reducing substances other than ascorbic acid by precipitation with mercuric acetate [Emmerie, 1934]. Van Eekelen [1934; 1935, 1] observed a normal amount of ascorbic acid in urine with a very high total reducing capacity (as determined by direct titration), the latter being chiefly due to an increased excretion of thiosulphate.

By evaluating during different diets the amounts of total reducing substances and also of ascorbic acid, conclusions have been reached which differ from those of Ahmad and Chakraborty & Roy.

EXPERIMENTAL.

Determination of ascorbic acid (and non-specific reducing substances).

The titrations were carried out in acid medium with 2:6-dichlorophenolindophenol [Tillmans *et al.*, 1932; Harris & Ray, 1933; Birch *et al.*, 1933; Johnson & Zilva, 1934], standardized against crystalline ascorbic acid.

As to the technique of eliminating interfering reducing substances by precipitation with mercuric acetate and reduction with H_2S we refer to Emmerie & van Eekelen [1934] and van Eekelen & Emmerie [1936].

The urine was both titrated directly and after treatment with mercuric acetate immediately after it had been voided. From the following table it may be observed that the reducing capacity may diminish even if the urine is kept with glacial acetic acid in the ice-box (as recommended by several investigators).

Direct titration:
1 ml. urine immediately 0·23; after 16 hours 0·17 ml. indicator
,, ,, 0·25 ,, 11 ,, 0·20 ,,
,, ,, 0·17 ,, 8 ,, 0·17 ,,
,, ,, 0·26 ,, 6 ,, 0·22 ,,

After precipitation with mercuric acetate:
Added immediately after voiding 10·0 ml. urine 1·32 ml. indicator
,, 6 hours ,, 10·0 ,, 1·08 ,,

Such a decline is the more important since the urine voided during the evening and during the night is generally kept for a considerable time and because particularly this urine is rather more concentrated (observed also by Harris *et al.* [1933]).

To determine the ascorbic acid in blood the method described by Emmerie & van Eekelen [1934] was used.

Diets. Two diets were used; the first contained 100 g. of rice with 50 g. of raisins, or 100 g. of spaghetti on alternating days, 600 g. of brown bread, 125 g. of butter, some honey, one egg and tea with sugar (per day). The second diet was composed of 750 g. beef, 6 eggs, 125 g. of butter, 400 g. of bread and tea per day. The caloric values of both diets were approximately equal (about 3200 kg.-cal.), the $\frac{\text{protein}}{\text{carbohydrate}}$ ratio was about $\frac{1}{8}\left(\frac{60}{500}\right)$ in the first, $\frac{1}{1}\left(\frac{217}{203}\right)$ in the second diet.

Before the experiments began a saturation test (*vide* Part II) was accomplished; the increased output in the urine following an intake of 500 mg. of "Redoxon" (pure ascorbic acid) (250 mg. on two successive days) indicates that the experimental subject was saturated.

Of the two diets the first is devoid of vitamin C, the second contains only those amounts present in beef (average 1·5 mg./100 g.). To avoid a too rapid decrease in the vitamin C content of the organism, a daily dose of 25 mg. of "Redoxon" was taken. In this way the vitamin C level in the blood did not become extremely low (7·2 mg./l.), nor did the urinary output of ascorbic acid decrease. Without a supply of vitamin C a more significant fall of the blood content had to be expected, demonstrating a depletion of the depots in the organism; the urinary output would have decreased likewise. In this way the experimental conditions might have been influenced so unfavourably, that an actual dietetic stimulation of the excretion of ascorbic acid could not become manifest.

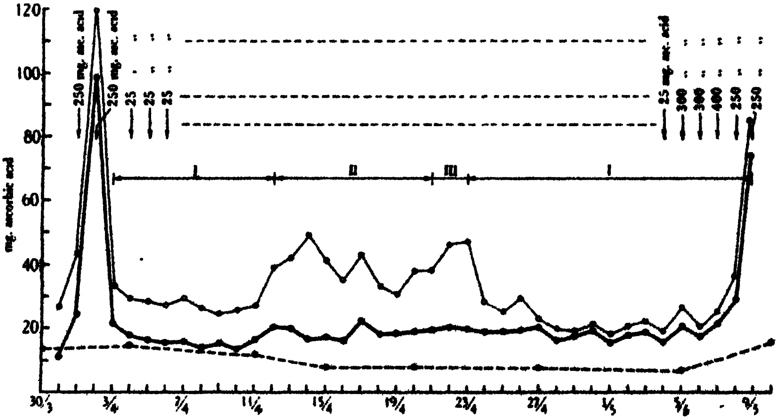

Fig. 1. Influence of proteins on the urinary output of non-specific substances and ascorbic acid. •— — —• mg./l. blood. •—• Daily urinary output of ascorbic acid. o—o Daily urinary output of total reducing substances (expressed as ascorbic acid). I = Low-protein diet. II = High protein diet. III = II without any carbohydrates.

The fact that the vitamin C level of the blood decreases in spite of the daily intake of 25 mg. of ascorbic acid is discussed later.

To be sure that the dietetic prescriptions were followed as strictly as possible and that any strain beyond the habitual work was avoided, the writer became the experimental subject. A control of the body weight showed no change during the experimental period.

The experiment demonstrates that during a diet rich in proteins the total reducing capacity of the urine increases. This increase originates mostly from a greater excretion of thiosulphate, as could be proved by its elimination with barium acetate.

Direct titration	After barium acetate	After mercuric acetate
11·30	3·88	3·62*

* Calculated as ascorbic acid in 286 ml. of urine.

The values obtained do not always agree so closely, for, like other investigators, we have observed that the ascorbic acid can be excreted partly in the reversibly oxidized form; in this case the reducing capacity of urine becomes greater after treatment with H_2S.

1 ml. urine	After H_2S	After barium acetate	After barium acetate and H_2S
0·095	0·13 (+0·035)	0·045	0·09 (+0·045)
0·115	0·15 (+0·035)	0·055	0·085 (+0·03)
1·62*	1·75 (+0·13)	1·11	1·23 (+0·13)

* While saturated with ascorbic acid.

The very distinct influence of a high protein diet on the total reducing capacity does not exist for the output of ascorbic acid. No definite rise in the excretion of ascorbic acid (determined after precipitation with mercuric acetate) has been observed. A certain increase between 10 and 12 April will be recognized as preceding the beginning of the protein diet. Furthermore, the somewhat higher level of the ascorbic acid excretion (after 11 April) continues after the protein content of the diet has been lowered, while the total reducing capacity decreases immediately.

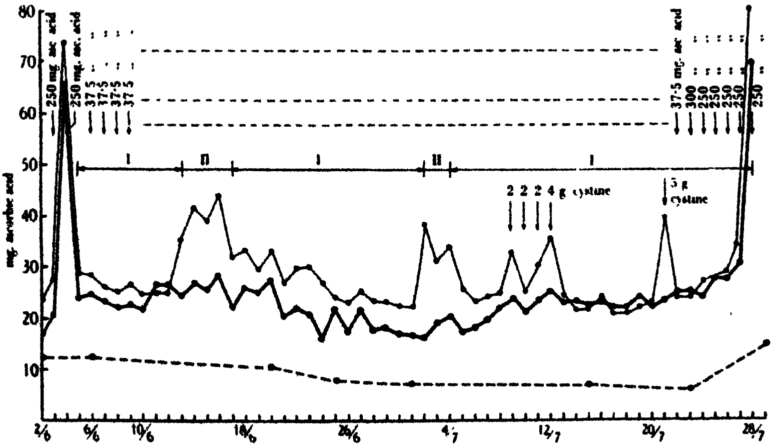

Fig. 2. Influence of protein and cystine on the urinary output of non-specific substances and ascorbic acid. •— — —• mg./l. blood. •—• Daily urinary output of ascorbic acid. o—o Daily urinary output of total reducing substances (expressed as ascorbic acid). I = Low protein diet. II = High protein diet.

After an intermediate period of 25 days of normal nutrition a second experiment under the same dietetic conditions was started. For reasons dealt with above a daily dose of vitamin C was taken, even larger than in the first experiment: 37·5 mg. instead of 25 mg. To get an insight in the cause of the influences exercised by proteins the latter were replaced by an amino-acid containing sulphur and taken in larger doses; cystine (Hoffmann-La Roche) was tried first.

Again we observe, that the high-protein diet increases the total reducing capacity and that the amount of ascorbic acid in the urine is not influenced. Cystine, given *per os*, increases the total reducing capacity in quite the same way. During intake of cystine and while on the beef-egg diet a greater diuresis was observed (compared with the carbohydrate regimen). The quantity of urine, however, did not influence the amounts of thiosulphate or ascorbic acid either (observed also by Johnson & Zilva [1934]). Large quantities of tea or water have been taken without any change in the amount of ascorbic acid or other reducing substances.

Date 1936	ml. urine	Ascorbic acid mg.	Reducing capacity expressed as ascorbic acid mg.
9. iv.	880	15·6	24·8
10. iv.	1530	13·8	25·8
11. iv.	1090	16·3	27·0
7. v.	1140	21·9	25·4
8. v.	1566	29·8	35·9
9. v.	937	74·5	84·4

Hawley *et al.* [1936] emphasize the possibility of an influence of *p*H on the amount of ascorbic acid in urine. We have not observed such an influence; moreover, the diets used were both acidotic. In the experiments of Chakraborty & Roy [1936] a high fat diet produced the same increase in the "total daily urinary excretion of ascorbic acid" as did the high protein diet. It will be seen that a suddenly augmented intake of butter had no such effect (20 July; 210 g. butter additional to the daily quantity of 125 g. were taken).[1]

PART II.

From measurements of the capillary resistance Göthlin [1931; 1932; 1934] concluded that about 25 mg. of ascorbic acid comprised the daily dose required for healthy adults weighing 60 kg. Referring to Stepp, Heupke [1936] gives an even smaller dose, viz. 10–20 mg. Stepp *et al.* [1936] now admit that this dose is "too low according to the most recent examinations". Diverging from this dose of 20–25 mg. van Eekelen [1935, 2; 1936, 1, 2] has found considerably greater requirements for man, his calculation being based on the results of a saturation test following a dietetic period devoid of vitamin C. The question being of practical importance a further contribution to it seemed desirable.

With regard to the results of Ahmad [1936] and Chakraborty & Roy [1936] and to the possible independence of requirements and urinary output (greater requirements with normal excretion?) two experiments were done on the same subject living on a diet devoid of vitamin C, but with different amounts of protein.

To ascertain the daily requirements the conditions of the experiments described in Part I were also suitable: a daily dose of ascorbic acid was taken equal to that postulated by Göthlin and formerly by Stepp; in the second experiment the dose was even larger.

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[1] Chopra & Roy [1936] likewise missed any influence of large amounts of fat on the output of total reducing substances, whereas the increase following high protein diet was confirmed.

EXPERIMENTAL.

Determination of requirements. The daily requirements are calculated according to the method of van Eekelen [1935, 2; 1936, 1, 2]. In the experiments with M. H. daily doses of 25 or 37·5 mg. of ascorbic acid were given during the dietetic period.

Table I.

I. Requirements while on a diet poor in protein (M. E.; male, aged 25, height 173 cm., 68·5 kg.).

	Ascorbic acid				Total reducing substances (expressed as ascorbic acid)	
Date 1936	Intake mg.	Blood mg./l.	Urinary output	Extra excretion (normal average about 17)†	Urinary output	Extra excretion (normal average about 25)†
Normal nutrition poor in vitamin C:						
10. vi.	250	13·64	17·30	—	26·88	—
11. vi.	250	—	32·88	—	39·95	—
12. vi.	—	—	97·7	—	113·04	—
Diet poor in protein, devoid of vitamin C:						
13. vi.	—	—	30·37	13	31·76	7
18. vi.	—	12·20	—	—	—	—
27. vi.	250	8·42*	—	—	—	—
28. vi.	250	—	—	—	—	—
29. vi.	250	—	15·84	—	24·66	—
30. vi.	250	—	48·22	31	53·71	28
18 days	1000	Minus		44†	or	35†

* Before "Redoxon" was taken.

II. Requirements while on a protein diet (M. E.).

				(Normal average about 20)†		(Normal average about 26)†
Protein diet devoid of vitamin C:						
1. vii.‡	—	—	17·54	—	28·41	—
2. vii.	—	14·15	22·26	—	28·79	—
3. vii.	—	—	16·87	—	23·59	—
4. vii.	—	—	18·90	—	30·40	—
5. vii.	—	13·86	—	—	—	—
8. vii.	—	10·0	—	—	—	—
11. vii.	—	8·4	—	—	—	—
21. vii.	—	7·49	—	—	—	—
22. vii.	300	—	22·95	—	27·12	—
23. vii.	300	—	23·16	—	32·40	6
24. vii.	300	—	30·79	10	33·70	7
25. vii.	300	—	65·98	46	70·31	44
25 days	1200 + 150 1350	Minus (25 × 6 mg. in 300 g. beef)		56†	or	57†

Calculation based on the excretion of:

	Ascorbic acid	Total reducing substances
Exp. I	$\frac{956}{18}=53{\cdot}1$ mg.	$\frac{965}{18}=53{\cdot}6$ mg.
Exp. II	$\frac{1294}{25}=51{\cdot}8$ mg.	$\frac{1293}{25}=51{\cdot}7$ mg.

† No complicated calculation is needed, the final result being not essentially influenced by it. Furthermore, differences up to 5 mg. in the daily required dose can and should be neglected as unimportant.

‡ This experiment following on the final saturation of the first one, further saturation could be omitted.

Diets. The diets prescribed were similar to those described in Part I, on account of the longer duration of the high protein period (25 days) the daily quantity of meat was 300 g. (instead of 750 g.).

The data do not need further commentary, we learn, however, that no difference exists as regards the usefulness of saturation tests between direct titration and that after treatment with mercuric acetate—at least so far as these experiments are concerned. The daily dose required in Exp. II is almost equal to that of the first one with a comparatively low proportion of proteins. Thus, a high protein diet failed to increase markedly the requirements of ascorbic acid as it did not influence its urinary excretion.

Requirements of M. H. (aged 37, height 174 cm., 75 kg. while on a daily dose of 25 mg. of ascorbic acid (see Fig. 1)).

Calculation based on the excretion of ascorbic acid:

Experimental period = 37 days. Ascorbic acid taken during the experimental period = 2213 mg. ("Redoxon") + 180 mg. (12 × 15 mg. in 750 g. beef) = 2393 mg. minus 76·5 mg. (excreted above the average) = 2316·5 mg.

Daily requirements $= \frac{2316 \cdot 5}{37} = 62 \cdot 6$ mg.

Calculation based on the excretion of total reducing substances

$$\frac{2393 - 75}{37} = 62 \cdot 6 \text{ mg.}$$

Requirements of M. H., while on a daily dose of 37·5 mg. of ascorbic acid (see Fig. 2).

Calculation based on the excretion of ascorbic acid:

Experimental period = 53 days. Ascorbic acid taken during the experimental period = 3312 mg. ("Redoxon") + 105 mg. (7 × 15 mg. in 750 g. beef) = 3417 mg. minus 63 mg. (excreted above the average) = 3354 mg.

Daily requirements $= \frac{3354}{53} = 63 \cdot 3$ mg.

Calculation based on the excretion of total reducing substances:

$$\frac{3417 - 67}{53} = 63 \cdot 2 \text{ mg.}$$

Daily doses of 25 or even 37·5 mg. of "Redoxon" failed to maintain the content of the blood at the level existing when the experiments began, nor did they prevent the development of a deficiency demonstrable in the course of saturation tests.

The subjects on whom the experiments were done were in good health; regarding the results of the saturation tests at the start of the experiments, their vitamin C conditions appeared to be normal.

DISCUSSION.

In the experiments described there has been no influence of high protein diets on the urinary output of ascorbic acid as suggested by Ahmad and Chakraborty & Roy. The different results are obviously caused by the fact that these authors did not remove interfering reducing substances. As to the total reducing capacity the observations agree, its increase by high protein diets having been confirmed. Furthermore, cystine exercises the same effect as does protein, and it appears very probable that not the proteins as a whole but rather their proportion of sulphur compounds causes the augmented reducing capacity. This point of view is further supported by the evidence that the excretion of thiosulphate is principally augmented. On the days of cystine intake sodium nitroprusside tests were constantly negative (absence of cysteine, which also decolorizes dichlorophenolindophenol [Emmerie, 1934]). Whereas this influence of high protein diets has been partly confirmed, a sudden and considerable rise in the proportion of fat was not found to exercise any influence.

Chopra & Roy [1936], who are apparently unacquainted with the elimination of interfering substances by mercuric acetate, suggest that the increase of the "indophenol titre" (following high-protein diet, direct titration without previous treatment) is not due to ascorbic acid. In fact, there could be demonstrated "a significant relation between this reducing property and the uric acid excretion". Uric acid, however, does not decolorize dichlorophenolindophenol.

The requirements of two normal subjects were calculated by determining the dose required for a positive saturation test after dietetic periods without any vitamin C or with an insufficient supply (25 and 37·5 mg. daily). Examinations during dietetic periods with different proportions of protein resulted in equal requirements in spite of or together with normal urinary output of ascorbic acid. The values found agree closely with those calculated by van Eekelen [1935, 2; 1936, 1, 2] and van Wersch [1936]. The daily requirements for a subject weighing 70 kg. amount to about 60 mg. This value being twice that given by Göthlin [1934], we tend to regard it as the optimum dose—the *actual requirement*; while the smaller one of Göthlin may be only preventive against the manifestation of scurvy—the *indispensable minimum*. This interpretation seems the more justified if we note that this author supposes 19–25 mg. to be "the smallest daily dose of ascorbic acid which, given *per os*, protects a person weighing 60 kg. against the slightest objectively ascertainable prescorbutic symptoms—those in the capillaries."

SUMMARY.

The urinary output of ascorbic acid, determined after precipitation with mercuric acetate, is not influenced by the proportion of protein in the diet.

The total reducing capacity of urine rises and falls corresponding to the quantity of protein in the food and depends chiefly on the output of thiosulphate.

Cystine, taken in the course of low protein diet, has the same effect on the total reducing capacity of urine as have large quantities of protein. The sulphur-containing amino-acids can be considered as the source of thiosulphate in urine.

By means of saturation tests following dietetic periods the daily human requirements were calculated.

Equal requirements were observed during low- and high-protein diets on the same subject.

The daily requirement of man amounts to about 60 mg. for a body weight of 70 kg. The same amount was required when 25 or even 37·5 mg. of "Redoxon" per day had been taken.

Considering this daily dose as *actual requirement*, the suggestion that an essentially smaller quantity is sufficient to avoid scurvy—the *indispensable minimum*—has been discussed and affirmed.

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CCCXX. THE INFLUENCE OF FERTILIZERS ON THE CAROTENE AND VITAMIN C CONTENT OF PLANTS.

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The estimation of carotene and vitamin C in vegetables has generally been carried out without paying attention to the different conditions prevailing during growth of the plants analysed. Therefore, the results obtained under these circumstances can only be looked upon as average values. Although these average values are sufficient for practical use, it is interesting, with a view to the quality of the crop, to examine if the carotene and vitamin C contents can be influenced by soil treatment and particularly by fertilizers.

The purpose of the investigations was to decide if the concentration of the elements necessary in plant-growth had any influence on the carotene and vitamin C contents of the plants.

EXPERIMENTAL.

The investigations were carried out by means of pot experiments, because only by this method of working can equal distribution of the salts be secured, this being essential to produce a crop of sufficient uniformity. In a number of the experiments the pots contained pure washed quartz sand, in other cases an exactly analysed sandy soil, which besides K and P deficiency also showed a low *p*H.

The pots were supplied with a salt solution according to Krüger of the following composition:

$MgSO_4, 7H_2O$	0·688 g.
KCl	0·552 g.
NH_4NO_3	0·752 g. or
$NaNO_3$	1·650 g.
Distilled water	1000 g.
$CaHPO_4, 2H_2O$	1·200 g.

$pH = 7{\cdot}2$

One or more components of this solution were omitted or administered in increased amounts, so that by the disturbance of the conditions of life an insight could be gained into the conditions of the formation of vitamins in plants.

K, Mg or N deficiency was produced by omitting the corresponding salt from the solution. In order to get Ca deficiency Na_2HPO_4 was used instead of $CaHPO_4, 2H_2O$ and PO_4 could be substituted by SO_4 to give P deficiency.

Pots of indifferent material were used, such as glass, enamelled iron and glazed earthenware.

Spinach was used as a test plant; it has the advantage of growing rapidly and containing considerable quantities of carotene and vitamin C.

The plants were kept in a glasshouse. The pots were weighed every day during the experiment and the loss in weight was compensated by the addition of distilled water.

The full-grown plants were cut with scissors. To avoid errors caused by differences in weight of the stalks, which contain only a very small quantity of ascorbic acid, the latter were discarded and only the leaves were analysed.

Table I.

	mg. of ascorbic acid per 100 g. of fresh material
Stalks	8
Leaves	19

The plants had to be analysed rapidly, because at room temperature and even at ice-box temperature the leaves show a rather rapid decrease in vitamin C content.

Table II. *Storage of spinach leaves in the ice-box (4°) in stoppered glass jars, with a piece of moist cotton-wool to keep the leaves fresh.*

No. of days of storage	mg. of ascorbic acid per 100 g.	
	Fresh small leaf	Fresh large leaf
0	35	46
1	29	43
3	4	18
5	5	8

Table III. *Storage of spinach leaves at room temperature and in the ice-box.*

No. of days of storage	mg. of ascorbic acid per 100 g.	
	Fresh leaf at room temp.	Fresh leaf in the ice-box
0	60	60
1	49	45
3	23	49
5	8	21

Methods.

The vitamin C content of leaves was determined by the titration method of Birch *et al.* [1933], with 2:6-dichlorophenolindophenol in acid medium.

The acid extract of fresh spinach leaves shows the same vitamin C content before and after treatment with H_2S or with mercuric acetate as used first by Emmerie [1934]. Therefore this method (Emmerie) has only been used when estimating the loss of ascorbic acid during storage of the leaves, because in this case it is possible that substances other than vitamin C are present which reduce the indicator or that, on the other hand, a certain amount of vitamin C is reversibly oxidized and cannot be determined by titration according to the method of Birch *et al.*

Carotene was extracted with ether, after saponification of the chlorophyll etc. by boiling the plant material with a saturated solution of KOH in 96% alcohol (20 ml. per g. of plant material) for half an hour [Guilbert, 1934]. Besides carotene and xanthophyll the ether contained saponified chlorophyll and flavones. The latter were removed by washing the ether with water. Then the ether was evaporated *in vacuo* and the residue dissolved in light petroleum; the xanthophyll was then removed from the light petroleum by extraction with 85 and 90 % alcohol.

This method can be simplified by extracting the carotene with light petroleum immediately after saponification. Thus, no chlorophyll or flavones and only a little of the xanthophyll dissolve in the light petroleum; the xanthophyll can be removed by washing once with 85 % methyl alcohol. The saponification must be carried out with only half of the amount of alcoholic KOH used by Guilbert. In this case, the concentration of the alcohol is reduced to about 90 % by the water in the plant material; at this concentration carotene dissolves sparingly and is extracted easily, while xanthophyll dissolves better in the alcohol than in the light petroleum. By 4 to 5 extractions all the carotene can be removed from the alcohol; further light petroleum extractions contain xanthophyll only, which can be washed out again with 85 % methyl alcohol. Therefore the extraction of carotene is complete when in the light petroleum extract all the yellow colouring matter can be removed with 85 % methyl alcohol.

The amount of carotene was determined with the Zeiss Stufenphotometer.

Results.

A. The influence of Ca, K, N and Mg deficiencies on the carotene and vitamin C contents of spinach. The experiment included 23 pots, filled with quartz sand.

Table IV.

Treatment	g. of leaf per pot	Type of leaf produced	Carotene γ g. of fresh leaf	Vitamin C mg./100 g. of fresh leaf
Ca deficiency	4	Normal	29	31
K deficiency	1	Small, dark, shrivelled margin	67	46
N deficiency	1·1	Small, light colour, erect	7	29
Mg deficiency	2	Light-coloured spots	34	31
Normal	4	—	31	33

B. The influence of added nitrogenous fertilizers on the carotene and vitamin C contents of spinach grown in pots filled with a sandy soil and supplied with increasing quantities of P_2O_5.

Each result is the mean of two analyses.

Table V. *Vitamin C in mg. per 100 g. of fresh leaf.*

Organic N mg. per pot	mg. P_2O_5 per pot				
	0	15	30	45	60
0	71	80	80	68	59
25	107	87	63	69	70
75	119	95	102	92	117
125	119	116	103	125	130

Table VI. *Carotene in γ per g. of fresh leaf.*

Organic N mg. per pot	mg. P_2O_5 per pot				
	0	15	30	45	60
0	26	30	26	33	26
25	65	38	33	44	41
75	71	77	63	65	63
125	95	87	75	71	87

Table VII. *Vitamin C in mg. per 100 g. of fresh leaf.*

Inorganic N mg. per pot	mg. P_2O_5 per pot				
	0	15	30	45	60
0	56	72	74	58	61
25	73	74	72	76	86
75	90	94	75	90	113
125	90	81	116	105	92

Table VIII. *Carotene in γ per g. of fresh leaf.*

Inorganic N mg. per pot	mg. P_2O_5 per pot				
	0	15	30	45	60
0	37	36	34	35	27
25	65	40	46	51	49
75	80	107	73	88	73
125	67	96	103	63	71

C. The influence of added nitrogenous fertilizers on the carotene and vitamin C contents of spinach grown in pots filled with a sandy soil and supplied with increasing quantities of potassium.

Each result is the mean of two analyses.

Table IX. *Vitamin C in mg. per 100 g. of fresh leaf.*

Organic N mg. per pot	mg. K_2O per pot			
	0	40	80	120
0	64	—	—	—
25	100	124	113	114
75	92	124	126	122
125	81	148	150	140

Table X. *Carotene in γ per g. of fresh leaf.*

Organic N mg. per pot	mg. K_2O per pot			
	0	40	80	120
0	100	—	—	—
25	124	86	45	49
75	93	101	94	104
125	100	92	82	94

Table XI. *Vitamin C in mg. per 100 g. of fresh leaf.*

Inorganic N mg. per pot	mg. K_2O per pot			
	0	40	80	120
0	—	—	—	—
25	—	105	119	122
75	121	121	136	134
125	—	70	142	140

Table XII. *Carotene in γ per g. of fresh leaf.*

Inorganic N mg. per pot	mg. K_2O per pot			
	0	40	80	120
0	99	—	—	—
25	113	86	62	65
75	80	106	96	81
125	113	104	106	113

D. Addition of phosphate had no effect.

E. From the results of the experiments recorded under *C*, the influence of added potassium fertilizer at different concentrations of nitrogen in the soil also becomes evident (Tables IX–XII).

DISCUSSION.

The results of the analyses show that the carotene and ascorbic acid contents of the test plants largely depend on the amounts of nitrogen and potassium in the soil.

A larger amount of nitrogen results in greater carotene and vitamin C contents.

An increasing potassium content of the soil causes a decrease in carotene and an increase in vitamin C. Diminution of the carotene content is only obvious in the plants grown in pots with little nitrogen. Generally the increase in vitamin C content is most distinct in plants grown with a high concentration of nitrogen. Under the conditions prevailing in the experiments, the influence of P, Ca and Mg salts is small.

Apart from the practical results, it is interesting to make an attempt to draw some physiological conclusions and to view the data in the light of what is known about the influence of fertilizers on photosynthesis and chlorophyll content.

Briggs [1922] was the first to investigate the influence of K, Mg and Fe. Gregory & Richards [1929] stated that the assimilation of nitrogen-deficient plants of *Hordeum* is "subnormal". Müller [1932] found the same for *Sinapis alba*. Gaszner & Goeze [1934] stated that larger additions of nitrogen resulted in an increased assimilation and transpiration and in increased chlorophyll and albumin contents both with rye and wheat; the difference between differently treated plants became greater when the plants grew older.

From the data of the experiments with N fertilizer described in this paper, it can be observed that nitrogen has an influence on the carotene and vitamin C contents similar to its influence on assimilation and chlorophyll content. It seems plausible to suppose that there exists a direct relationship between photosynthesis and vitamin C content, in view of the fact that the vitamin C content of leaves increases when they are irradiated with neon-light and that etiolated plants contain no vitamin C. Another argument in support of this theory can be found in the experiment of Randoin *et al.* [1935], who found less vitamin C in white than in green portions of plants.

This connexion with photosynthesis does not hold good in the case of carotene. Etiolated plants show no assimilation and possess carotene. Moreover Willstätter & Stoll [1913], Scherz [1929], Pfützer & Pfaff [1935] state that the carotene content of plants fluctuates with the chlorophyll content. Furthermore, Karrer & Helfenstein [1931] pointed out that carotene is derived from phytol or phytolaldehyde, establishing a chemical relationship between the nitrogenous chlorophyll and the N-free carotene.

From the data concerning the influence of nitrogenous fertilizer it seems that there exists a relationship between photosynthesis and vitamin C content on the one hand and between chlorophyll content and carotene on the other.

The data obtained from the experiments with potassium fertilizer confirm this supposition.

Gaszner & Goeze [1934] find an increased photosynthesis under the influence of more potassium fertilizer in wheat and rye, 25 days old and grown in a medium of comparatively high N content. Plants grown with little N show practically no difference in assimilation under influence of K fertilizer.

At a low N level the chlorophyll content of K-deficient plants is much higher than of plants richly supplied with potassium; at a high N level this difference does not exist.

Concerning the influence of potassium on photosynthesis, similar results were obtained by Briggs [1922], Gregory & Richards [1929] and Lundegårdh [1932]; the decrease of green colouring matter as a result of increasing quantities of K fertilizer is a well-known fact to every farmer and is frequently mentioned in the literature [see Maiwald, 1923; Remy & Dhein, 1932; Remy & Liesegang, 1926].

From the data concerning the influence of K fertilizer on the carotene and vitamin C contents of spinach, grown on a sandy soil with increasing quantities of nitrogen, it can be readily seen that carotene shows the same fluctuations as chlorophyll, whereas obviously vitamin C is a product of assimilation or at least closely connected with the process. Carotene content decreases at low N levels as a result of increasing quantities of K, whereas this decrease is practically zero at high N levels; vitamin C content increases but little at low N levels as a result of the addition of K fertilizers, whereas at high N levels a rapid increase can be observed.

From the results of the experiments it can also be concluded that nitrogen and potassium stand in close interrelation physiologically; K deficiency has the effect of N excess and K excess acts like N deficiency.

Therefore, fertilizer experiments concerning one of these two elements only give accurate results if made at different concentrations of the other element.

SUMMARY.

1. Details are given of the methods of growing spinach for analysis.

2. Tables are presented showing loss in vitamin C content during storage.

3. A modified method for estimating the carotene content of vegetables is given.

4. Tables are presented showing the influence of K, N, Ca and Mg fertilizers on the carotene and vitamin C contents of spinach. A higher level of nitrogen results in a greater carotene and vitamin C content. An increasing potassium content of the soil causes a decrease in carotene content and an increase in vitamin C.

5. An attempt is made to show a relationship between chlorophyll and carotene; on the other hand the suggestion that ascorbic acid only can be a product of photosynthesis is discussed.

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CCCXXI. AN INVESTIGATION OF THE RATES OF DIGESTION OF STARCHES AND GLYCOGEN AND THE BEARING ON THE CHEMICAL CONSTITUTION.

II. LIVER AMYLASE.

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Great diversity of opinion exists as to the nature of the end-products and the course of hydrolysis of glycogen by liver amylase.

Macleod [1926] stated that the final product was maltose which was, however, liable to be hydrolysed further to glucose. Osborne & Zobel [1903], working on muscle juice, found that maltose was the chief end-product together with a trace of glucose. Hollander [1934], using rat liver preparations and starch as substrate, found maltose as the sole end-product. He arrived at the conclusion that the liver preparations contain two amylases comparable with α- and β-plant amylases. Tebb [1894; 1897], using glycogen and a dried pig liver preparation, found glucose as the sole product. Preliminary saline perfusion of the liver through the portal vein, to remove all the blood, and subsequent drying provided a liver preparation which still had marked maltase activity. Hodgson [1936], using acetone-extracted and dried rabbit liver preparations, claimed, it appears without satisfactory experimental evidence, that glucose was the sole end-product and was unable to detect the presence of maltose at any stage of hydrolysis. These results are referred to again later. Barbour [1929], who used glycerol extracts of rabbit muscle, claimed to have obtained a trisaccharide as the sole product of hydrolysis. This trisaccharide is stated to have a reducing power 30 % of that of glucose (by Shaffer-Hartman method), a specific rotation $[\alpha]_D$ 181°, to be readily transformed into an anhydride and to form an osazone crystallizing in small star-shaped aggregates of needles. This trisaccharide is not identifiable with Pringsheim's [1924] amylotriose, obtained by the breakdown of glycogen and amylopectin with concentrated HCl. Lohmann [1926], using KCl extracts of frog muscle, similar to those used by Meyerhof [1926] studying simultaneously the production of lactic acid by glycogen, describes, in addition to glucose, the formation of a trisaccharide which he considers to be probably identical with Pringsheim's amylotriose. Case [1931], without sufficient experimental data, claims to have repeated and confirmed Barbour's results. Carruthers & Wei Yung-Lee [1935] also repeated Barbour's work, but contrary to this worker found that maltose, and not a trisaccharide, was the chief product of hydrolysis. They also obtained evidence of the formation of some glucose. Carruthers [1935] explained Barbour's results as due to inhibition of the maltase activity of his preparations by the glycerol employed in the extraction.

In the present investigation liver preparations from the cat, rat, rabbit, pig, ox, guinea-pig and frog were employed. The preparations were made according to the method suggested to me by Prof. J. Mellanby and as used and described by Hodgson [1936]. Eadie [1927] pointed out the necessity for perfusing the liver to remove all blood, which contains some amylase, before testing the amylolytic activity of the liver preparation. Consequently a similar preparation was made from the liver of a rat which had been perfused until blood-free. In all cases the maltase activity of the dried liver preparations was tested by incubating 0·5 g. of the liver preparation with 25 ml. 2 % maltose solution, after addition of a few drops of toluene, for 24 hours at 37°. The reducing power of the digest was determined by Hanes's method after suitable preliminary dilution and precipitation of proteins with "colloidal iron" and acetic acid.

An osazone was also made. In all the cases tested (the perfused liver preparation which was all employed in the glycogen hydrolysis was not tested) a considerable amount of glucose was formed, showing that all these preparations contain maltase. It must be emphasized, however, that the amount of liver preparation employed in this test was relatively very much greater than that used in the subsequent hydrolyses. The capacity of these preparations to form glucose from maltose might be due to the maltase activity of the liver tissue itself or to the maltase activity of the blood in the preparation. (A comparison of the hydrolysis by the unperfused and perfused rat liver preparations is of significance in this connexion.) The maltase activity of the serum of the rat, rabbit, cat and ox was tested in a similar manner to that described above, in this case employing 5 ml. serum. In the case of the rat serum, there was a quantitative conversion of maltose into glucose, an almost quantitative conversion with the ox serum, whereas both cat and rabbit serum showed no maltase activity. These results are in general accord with those of Barnes & Mackay [1936]. Hynd & Macfarlane [1927], determining the maltase activity of whole blood, found that pig's blood alone gave positive results and obtained negative results with the blood of the mouse, rat, guinea-pig, rabbit, kitten, ox and sheep. Tebb [1894] also found that pig serum had marked maltase activity.

Course of digestion of glycogen by liver amylase.

Digestion mixtures were set up containing 1·0 g. glycogen (Kahlbaum) in 160 ml. 0·1 % NaCl, 40 ml. phosphate buffer *p*H 6·4, 1·0 g. liver preparation and 1 ml. toluene.

A control mixture was set up omitting the glycogen. The hydrolysis of potato starch was also followed in some cases. The digestion mixtures were placed in an incubator at 37° and samples removed, boiled, filtered and analysed at intervals up to 3 days. To test Carruthers's criticism of Barbour's work, 0·25 g. glycogen dissolved in 30 ml. 0·1 % NaCl, 10 ml. glycerol, 10 ml. phosphate buffer *p*H 6·4 and 0·25 g. pig liver preparation were incubated for 17 hours.

The following estimations were performed:

(1) Unchanged polysaccharide.
(2) Total reducing power.
(3) Reducing power due to sugar alone.
(4) Reducing power after acid hydrolysis.
(5) Lactic acid.

The unchanged polysaccharide was estimated as described for glycogen in a previous paper [Glock, 1936]. The sugar determinations in each case were performed according to the ferricyanide method of Hanes [1929]. The total reducing power was determined on 5 ml. samples of a 1 in 10 dilution of the digest.

A further 10 ml. of the digest was diluted to 50 ml. with absolute alcohol, to precipitate any unchanged glycogen and dextrins, left for 2–3 hours or longer and filtered. Two 20 ml. portions of the filtrate were taken and the alcohol removed by evaporation. The reducing power of one portion was determined directly after suitable dilution and of the other after acid hydrolysis followed by subsequent neutralization and dilution. 20 ml. of the digest were employed for the estimation of lactic acid, by the method of Friedemann & Graeser [1933]. Lactic acid determinations were not made in the early stages of hydrolysis. In each case an osazone was made after alcohol precipitation and subsequent evaporation of the alcohol. The results are represented diagrammatically in Fig. 1. The reducing sugar for convenience is calculated and expressed as "maltose" although in some cases the sugar was known to consist partly or entirely of glucose.

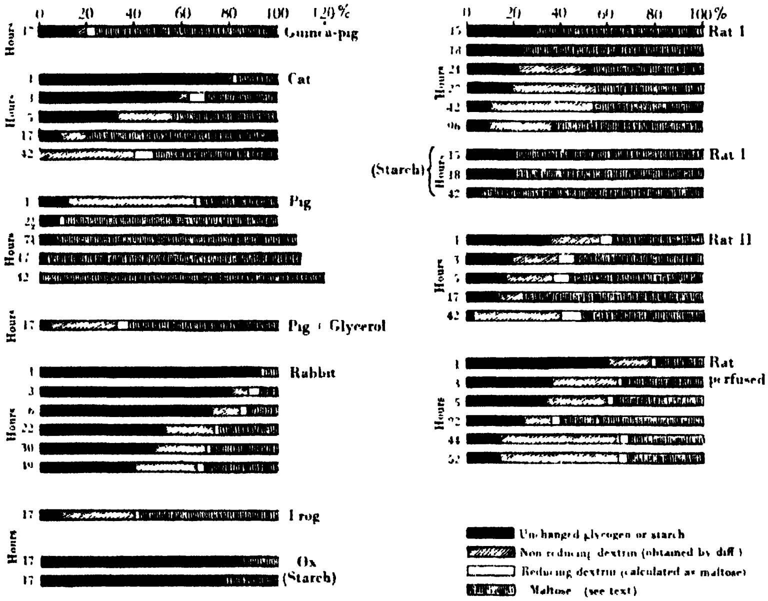

Fig. 1.

DISCUSSION OF RESULTS.

From Fig. 1 it appears that in the breakdown of glycogen to sugar there is no appreciable formation of reducing dextrin, since the reducing power of the digest after alcohol precipitation of dextrins and unchanged glycogen is only very slightly different from that before precipitation. It would seem, therefore, that in the hydrolysis by liver amylase, sugar is chiefly split off successively from the glycogen molecule and is only formed to a much smaller degree from simple dextrins produced intermediately. In this respect the action of liver amylase is different from those of salivary, malt and pancreatic amylases.

From the results of the hydrolysis of potato starch by liver amylase it is seen that starch is broken down rather more easily than glycogen, but this difference between the rates of breakdown of the two polysaccharides by liver amylase is not nearly as marked as with salivary, pancreatic and malt amylases.

In all cases the formation of lactic acid was insignificant, the amount produced varying from 0·87 to 4·76%.

Three different types of osazone were observed. The first was the "*iso*-maltose" type consisting of relatively large rosettes of very fine needles, sometimes tufted at their free extremities. This type of osazone was observed in the early stages of hydrolysis with all the liver preparations and throughout the hydrolysis with the cat, rabbit and perfused rat liver preparations. with the pig liver preparation in the presence of glycerol and generally in addition to glucosazone in the later stages of hydrolysis with the rat liver preparation. The second type of osazone (or hydrazone?) was one not previously observed. It consisted of large pale yellow fern-like crystals. Owing to their pale yellow colour and to the fact that they were relatively insoluble in hot water, it was considered possible that the crystals might be those of a hydrazone and not of an osazone. This osazone was generally found after 42 hours' hydrolysis with the cat and rat liver preparations. The third type of osazone, glucosazone, was the only product formed by the pig liver preparation after 17 hours and longer and was also formed by the rat liver preparation, but in the latter case an additional osazone was always obtained.

The results of acid hydrolysis corroborate the view that glucose is the sole product of hydrolysis with the pig liver preparation. The first type of osazone is probably produced by maltose. perhaps modified in crystalline form by admixture with unprecipitated dextrin, since the amount of glucose formed on acid hydrolysis in the later stages of hydrolysis was 100-110% of the theoretical yield.

If graphs are plotted of the percentage formation of sugar calculated as maltose against time it is seen that with the pig and rabbit liver preparations the formation of reducing sugar follows a normal hyperbola. whereas this is not true with the rat (perfused and unperfused) and cat liver preparations. In the latter cases there is a sudden fall in the reducing power which finally rises slowly after 42 hours. This type of hydrolysis curve would appear to be due to the reversible action of the liver amylase, the breakdown phase being predominant in the early stages of digestion and the synthetic phase in the later stages, probably initiated by accumulation of the hydrolysis products of the breakdown phase. The possible criticism that the decrease in the reducing power in the later stages might be due to the formation of hexosephosphates is disproved by the fact that a similar type of curve was obtained using the rat liver preparation but replacing the phosphate buffer by a citrate buffer of the same *p*H. This decreased reducing power also cannot be accounted for by increased lactic acid formation, for in no case was the percentage formation greater than 5.

It is interesting to note, in this connexion, that the third type of osazone described above was only observed in the 42 hours' digest with the rat and cat preparations and is, therefore, probably correlated with the decreased reducing power. The breakdown of potato starch by rat liver preparations, however, follows a normal course.

The conversion of glycogen into glucose by liver preparations depends on the concentration of amylase and maltase. If both enzymes are very active, it is possible to get 100% conversion into glucose: this is the case with the pig liver preparation. If the concentration of either amylase or maltase is reduced, the yield of glucose will be less: this seems to be the case with the rat preparation. If the concentration of maltase is very small, the breakdown will stop at the maltose stage and will not proceed as far as glucose. It was shown, as already described, that all the preparations possessed maltase activity. In the case of the cat and rabbit preparations this activity must have been much less than that of the pig and rat preparations, so that in the amount of preparation which was used in the glycogen hydrolysis, the maltase concentration was negligible. If the maltase activity of the preparation were due to the blood it contained, then one would suppose that after perfusion of the liver to render it blood-free, the breakdown of glycogen would not proceed further than the maltose stage. This was found to be true with the dried preparation from the perfused rat liver.

That the end-product in this case was not glucose was further substantiated by the fact that after alcohol precipitation and subsequent evaporation of the alcohol, the liquid was not fermented by a yeast known to be incapable of fermenting maltose. In the case of the rat, the maltase activity of the serum was found to be so great that the ability of the unperfused liver preparation to form glucose might easily be attributed to the maltase activity of the blood. It is doubtful, however, whether this is true of the pig liver preparation since in this case there was normally 100 % conversion into glucose. (That perfused pig liver preparations still possess marked maltase activity has been shown by Tebb [1894].) The breakdown of glycogen would also stop at the maltose stage if the maltase activity of the preparation were inhibited, for example, by the method of preparation. The pig liver preparation, in the presence of glycerol, failed to produce any glucose after 17 hours This also supports Carruthers's opinion of Barbour's work.

From the values of the reducing power before and after acid hydrolysis, the relative amounts of glucose and maltose at each stage of digestion can be calculated. At the end of 17 hours the sugar formed consisted in the case of the pig preparation of 16 % maltose and 84 % glucose and in the cases of the rat, guinea-pig and ox liver preparations of 93 % maltose and 7 % glucose, 91 % maltose and 9 % glucose and 79 % maltose and 21 % glucose, respectively. After 42 hours the values were for the pig preparation 100 % glucose, for the rabbit 100 % maltose and for the rat 88 % maltose and 12 % glucose. The necessity for estimating the reducing power both before and after acid hydrolysis in order to form an opinion as to the nature of the sugar produced is obvious when the results obtained with the pig preparation are considered: after 7½ hours only 15 % of the sugar was glucose, after 17 hours 84 %, whereas at 42 hours there was a 100 % yield.

A final 100 % yield of glucose does not necessarily mean that no maltose is produced intermediately. Hodgson [1936], without having performed any acid hydrolyses, claims that glucose is the sole product of hydrolysis of glycogen by rabbit liver preparations. It is obvious from the above results that such an assumption is unfounded.

SUMMARY.

1. The hydrolysis of glycogen, and in a few cases of potato starch, by acetone-extracted and dried liver preparations of the rat, cat, rabbit and pig in the presence of phosphate buffer of pH 6·4, was followed. The course of hydrolysis of glycogen was also followed using a perfused rat liver preparation and in addition the behaviour of the pig liver preparation in the presence of glycerol was studied.

2. The maltase activity of each liver preparation was tested, in all cases with a positive result. The maltase activity of the serum was also tested. That of the rat serum was found to be very great, that of the ox quite large, whereas cat and rabbit sera possessed no maltase activity.

3. In the case of the pig liver preparation only was there quantitative conversion into glucose. This formation of glucose is inhibited by the presence of glycerol.

4. Maltose was the sole end-product with the cat, rabbit and perfused rat preparations.

5. The unperfused rat liver preparation produced maltose alone in the early stages of hydrolysis, but this was gradually converted into glucose as digestion proceeded.

6. In the cases of the rat (perfused and unperfused) and cat liver preparations the reducing power showed a steady decrease from 17 to 42 hours. This can only be explained by a reversal of the enzymic activity.

8. That the production of glucose by the rat liver preparation could be due to the maltase contained in the blood has been proved since no glucose was produced by the perfused rat liver preparation.

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CCCXXII. KETOGENESIS-ANTIKETOGENESIS.

V. METABOLISM OF KETONE BODIES.

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Acetoacetic and β-hydroxybutyric acids are known to undergo two chief metabolic changes, interconversion and oxidative breakdown. The reversibility of the reaction, acetoacetic acid $\rightleftharpoons$ β-hydroxybutyric acid, was demonstrated in liver brei by Dakin & Wakeman [1910, 1, 2] and in perfused liver by Friedmann & Maase [1910]. Furthermore, the interconversion of these keto- and hydroxy-acids, both in liver and in kidney, was clearly illustrated by the perfusion experiments of Snapper & Grünbaum [1927, 1, 2, 3]. Using slices, Jowett & Quastel [1935] have shown that β-hydroxybutyric acid is oxidized to acetoacetic acid in tissues other than liver and kidney.

Apart from the interconversion Snapper & Grünbaum found a true destruction of "ketone bodies", which was large in kidney but very small in liver. β-Hydroxybutyric acid was also destroyed in the extremities of the dog and by the tongue muscles of the calf [Snapper & Grünbaum, 1928].

Quastel & Wheatley [1935] studied the breakdown of acetoacetic acid in kidney slices. Acetoacetic acid disappeared under both aerobic and anaerobic conditions: in the former instance only one-quarter to one-third of the change was due to reduction to β-hydroxybutyric acid, whereas in the latter reduction was almost quantitative. The distinct nature of the two processes was demonstrated by the action of inhibitors, e.g. malonate.

Methods.

Various tissues of rats, guinea-pigs and pigeons were used in this work. Slices of lung, pancreas, submaxillary gland and skeletal muscle were cut by the method of Deutsch [1936]. Lung slices float on cold Ringer solution but sink into the medium as soon as the containing vessel is transferred to a thermostat at 37·5°. The quantities of tissue employed in manometric experiments were: kidney and brain, 8–10 mg. (dry weight), other tissues 10–20 mg. (dry weight). Slices of striped muscle were suspended in a "kochsaft" made from pigeon's breast muscle and buffered to *p*H 7·4 with phosphate according to the method of Krebs (unpublished).

Aerobic experiments. Tissue respiration was measured in Warburg manometric vessels of the conical type, the medium being 2–3 ml. phosphate saline [Krebs, 1933]. The vessels were filled with O_2 and shaken in a thermostat at 37·5°. At the end of 2 hours the slices were removed and the change in ketone bodies, either formation or disappearance, was determined by methods already described [Edson, 1935].

Determination of ketone bodies. Acetoacetic acid was usually determined manometrically by the aniline citrate method, but if β-hydroxybutyric acid was to be estimated simultaneously it was more convenient to apply the modified Van Slyke procedure. The method of Ostern [1933] was employed when mesoxalic acid was present, since the aniline citrate method is inaccurate under such conditions. β-Hydroxybutyric acid was estimated by the modified Van Slyke method.

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We have found that certain substances interfere with the Van Slyke determinations.

1. Pyruvate gives a large quantity of insoluble mercury compound during the first boiling (30 min.) with Denigès' reagent. In presence of pyruvate it is impossible to determine acetoacetic acid as mercury-acetone compound, but since the whole of the pyruvate reacts during the first boiling, it is possible to remove the precipitate and proceed to a determination of β-hydroxybutyric acid. Control experiments with pyruvate have shown that there is no further precipitation on heating for 90 min. after addition of dichromate, and that β-hydroxybutyric acid can be determined satisfactorily in solutions which originally contained pyruvate. If simultaneous determinations of both ketone bodies in presence of pyruvate are required, acetoacetic acid must be estimated manometrically in a separate sample.

2. Crotonic acid reacts with Denigès' reagent during both steps of the Van Slyke method. If the precipitate is considered to be wholly acetone-Hg compound, 1 mol. crotonic acid gives 0·33 mol. acetone.

3. 2 ml. malonic acid solution, 0·02 *M*, react to a slight extent with Denigès' reagent during the first boiling. A satisfactory blank correction is readily made. 2 ml. malonic acid solution, 0·01 *M*, or 2 ml. hydroxymalonic acid solution, 0·01 and 0·02 *M*, give no mercury precipitates under the conditions of the determination.

Anaerobic experiments. In examining the anaerobic disappearance of ketone bodies we used the bicarbonate-Ringer solution of Krebs & Henseleit [1932] in equilibrium with 5 % CO_2 and 95 % N_2. Anaerobic acid production was measured during the course of 2-hour experiments at 37·5°.

Larger scale experiments. In some experiments it was necessary to use greater amounts of tissue (60 mg. dry weight) and of Ringer solution (10 ml.). The shaking was then performed in the large vessels described by Krebs [1933]. 2 ml. samples were extracted for duplicate determinations of ketone bodies.

Units. The rate of metabolism is expressed in the usual gas notation by the following quotients:

Q_{O_2} = μl. O_2 (N.T.P.) consumed per mg. dry weight of tissue per hour.

Q_{Acac} = μl. CO_2 (N.T.P.) β-ketonic acid formed per mg. dry weight of tissue per hour. 1 millimol β-ketonic acid = 1 millimol CO_2.

$Q_{\beta\text{-Hydroxy}}$ = μl. CO_2 (N.T.P.) β-hydroxybutyric acid formed per mg. dry weight of tissue per hour. 1 millimol β-hydroxybutyric acid = 1 millimol CO_2.

EXPERIMENTAL.

Anaerobic disappearance of acetoacetic acid in animal tissues.

Quastel & Wheatley [1935] have shown that kidney slices reduce large amounts of acetoacetic acid to β-hydroxybutyric acid when the conditions are virtually anaerobic (respiration poisoned with HCN).

The experiments of Table I were performed with the object of comparing the rates of anaerobic disappearance of acetoacetic acid in different tissues and in presence of substrates which might be expected to affect the process. Preliminary work led us to attach particular importance to pyruvate and fructose.

Table I. *Anaerobic disappearance of acetoacetic acid in presence of tissue slices.*

2–3 ml. Bicarbonate-Ringer solution, *p*H 7·4. 2 hours at 37·5°. Gas: 5% CO_2 and 95% N_2.

Exp.	Animal	Substrate	$Q^{N_2}_{CO_2}$	Q_{Acac}
		M initial concentration		
		Liver.		
1	Rat	Acetoacetic acid, 0·0033	3·14	– 1·68
		„ + glucose, 0·01	2·92	– 1·83
		„ + glycerol, 0·01	2·73	– 2·08
		„ + sorbitol, 0·01	2·86	– 2·00
		„ + lactate, 0·01	3·57	– 2·38
		„ + pyruvate, 0·01	6·40	– 2·66
		„ + glyceraldehyde, 0·01	5·86	—
		„ + alanine, 0·01	3·39	– 2·12
2	Rat	Pyruvate, 0·01	3·16	0·00
		„ 0·02	3·22	0·00
		Glyceraldehyde, 0·01	4·68	0·00
		Acetoacetic acid, 0·0033	2·31	– 2·31
		„ + pyruvate, 0·01	5·36	– 3·78
		„ + pyruvate, 0·02	5·20	– 3·03
		„ + glyceraldehyde, 0·01	5·57	—
		„ + glucose, 0·01	2·25	– 1·81
3	Rat	Acetoacetic acid, 0·0033	1·99	– 1·06
		„ + glucose, 0·01	1·57	– 0·98
		„ + galactose, 0·01	1·42	– 1·07
		„ + mannose, 0·01	1·72	– 1·35
		„ + fructose, 0·01	5·46	– 2·49
		„ + pyruvate, 0·01	5·98	– 2·33
		„ + lactate, 0·01	2·25	– 1·26
		„ + pyruvate, 0·01 + glucose, 0·01	7·75	3·05
4	Rat	Acetoacetic acid, 0·0023	1·88	– 1·04
		„ + pyruvate, 0·01	5·22	– 2·18
		„ + fructose, 0·01	3·42	– 2·42
		„ + fructose, 0·01 + pyruvate, 0·01	5·85	– 2·56
5	Rat	Acetoacetic acid, 0·0033	1·93	– 1·27
		„ + hexosediphosphate, 0·01	2·77	– 1·53
		„ + β-phosphoglycerate, 0·01	1·62	– 1·14
		„ + α-phosphoglycerate, 0·01	2·15	– 1·41
		„ + α-glycerophosphate, 0·01	2·24	– 1·99
		„ + butyrate, 0·01	1·61	– 1·08
		„ + acetate, 0·01	2·08	– 1·67
		„ + *n*-valerate, 0·01	2·10	– 1·41
6	Rat	Acetoacetic acid, 0·0033	1·49	– 1·92
		„ + methylglyoxal, 0·005	4·40	—
		„ + methylglyoxal, 0·0025	3·44	—
		„ + acetaldehyde, 0·01	4·02	– 2·40
		„ + acetaldehyde, 0·005	2·80	– 2·57
		„ + 1 mg. glutathione	1·20	– 1·93
		„ + malonate, 0·01	1·01	– 1·76
7	Rat, starved 24 hours	Nil	1·40	+ 0·14
		Fructose, 0·01	1·80	+ 0·05
		Acetoacetic acid, 0·0023	1·57	– 0·71
		„ + fructose, 0·01	2·34	– 1·61
		„ + glucose, 0·01	1·70	– 0·88
		„ + pyruvate, 0·01	3·95	– 2·33
		„ + pyruvate, 0·001	2·09	– 1·28
		„ + glucose, 0·01 + pyruvate, 0·01	4·38	– 1·96
		„ + glucose, 0·01 + pyruvate, 0·001	4·46	– 1·69

Table I (*cont.*).

Exp.	Animal	Substrate *M* initial concentration	$Q_{CO_2}^{N_2}$	Q_{Acac}
		Kidney cortex.		
8	Rat	Acetoacetic acid, 0·0033	1·90	– 1·50
		,, + glucose, 0·01	5·03	1·78
		,, + glycerol, 0·01	1·67	1·55
		,, + pyruvate, 0·01	4·25	- 1·85
		,, + alanine, 0·01	1·88	2·62
		,, + lactate, 0·01	2·32	– 1·55
		,, + cysteine, 0·01	1·75	1·23
9	Rat	Acetoacetic acid, 0·0033	2·00	- 1·39
		,, + pyruvate, 0·02	3·92	– 1·44
		,, + hexosediphosphate, 0·01	2·41	– 1·35
		,, + α-phosphoglycerate, 0·01	2·43	– 1·79
		Pyruvate, 0·02	3·06	+ 0·01
10	Rat	Acetoacetic acid, 0·0033	1·74	- 1·63
		,, + α-glycerophosphate, 0·01	1·80	– 1·90
11	Rat	Acetoacetic acid, 0·0033	1·65	- 1·34
		,, + fructose, 0·01	2·07	- 1·33
		Fructose, 0·01	1·86	0·00
12	Rat	Acetoacetic acid, 0·0033	1·58	- 1·56
		,, + sorbitol, 0·01	1·37	1·56
		,, + glyceraldehyde, 0·01	4·10	—
		,, + succinate, 0·01	1·07	– 1·55
		,, + glutarate, 0·01	1·73	1·41
		,, + malonate, 0·01	1·61	1·35
		,, + propionate, 0·01	1·70	1·21
13	Guinea-pig	Acetoacetic acid, 0·0033	1·69	- 1·26
		,, + fructose, 0·01	2·70	- 1·80
		,, + pyruvate, 0·01	3·24	- 1·47
		,, + alanine, 0·01	1·54	1·79
		,, + 1 mg. glutathione	1·72	1·52
14	Pigeon	Acetoacetic acid, 0·0033	1·41	- 1·69
		,, + pyruvate, 0·01	4·00	– 2·32
15	Pigeon	Acetoacetic acid, 0·0033	1·43	- 1·04
		,, + pyruvate, 0·01	4·02	2·25
		Testis.		
16	Rat	Acetoacetic acid, 0·0033	1·76	- 1·33
		,, + pyruvate, 0·01	3·78	– 1·18
		,, + glyceraldehyde, 0·01	3·08	-
		,, + glucose, 0·01	5·06	– 1·57
		,, + alanine, 0·01	1·45	– 1·20
17	Rat	Acetoacetic acid, 0·0033	2·61	– 1·24
		,, + fructose, 0·01	3·92	1·12
		,, + α-glycerophosphate, 0·01	2·44	– 0·83
		Brain cortex.		
18	Rat	Acetoacetic acid, 0·0033	0·92	– 1·19
		,, + pyruvate, 0·01	3·05	0·94
		,, + fructose, 0·01	0·94	– 0·66
19	Guinea-pig	Acetoacetic acid, 0·0033	1·07	- 0·71
		,, + pyruvate, 0·01	2·34	- 0·87
		,, + fructose, 0·01	2·14	- 1·01
		,, + glucose, 0·01	21·2	- 1·15
		Glucose, 0·01	20·3	0·00
20	Guinea-pig	Acetoacetic acid, 0·0033	1·08	- 0·86
		+ pyruvate, 0·01	2·68	- 0·51
		,, + fructose, 0·01	1·95	– 0·42

Table I (*cont.*).

Exp.	Animal	Substrate *M* initial concentration	$Q_{CO_2}^{N_2}$	Q_{Acac}
		Brain cortex (*cont.*). (1 hour only)		
21	Guinea-pig	Acetoacetic acid, 0·0023	2·53	−2·07
		„ + glucose, 0·01	22·1	2·28
		„ + glyceraldehyde, 0·01	2·96	
		„ + α-glycerophosphate, 0·01	1·80	−4·75
22	Pigeon	Acetoacetic acid, 0·0033	1·73	−1·97
		„ + pyruvate, 0·01	2·66	−1·73
		„ + fructose, 0·01	1·51	−1·63
		Spleen.		
23	Rat	Acetoacetic acid, 0·0033	1·52	−1·04
		„ + pyruvate, 0·01	2·23	−1·04
		Intestine.		
24	Rat	Acetoacetic acid, 0·0033	4·60	−1·04
		„ + pyruvate, 0·01	5·50	0·84
		„ + fructose, 0·01	4·98	−0·85
		Pancreas.		
25	Rat	Acetoacetic acid, 0·0023	1·99	−0·52
		„ + pyruvate, 0·01	2·81	−0·35
		„ + fructose, 0·01	2·47	−0·34
26	Pigeon	Acetoacetic acid, 0·0023	1·88	1·05
		„ + pyruvate, 0·01	2·64	−1·25
		Submaxillary gland.		
27	Guinea-pig	Acetoacetic acid, 0·0033	0·85	−0·27
		„ + pyruvate, 0·01	1·58	0·43
		Diaphragm.		
28	Rat	Acetoacetic acid, 0·0033	1·08	0·48
		„ + hexosediphosphate, 0·01	1·55	−0·45
		„ + pyruvate, 0·01	1·74	−0·63

Sodium acetoacetate solutions were prepared according to Ljunggren [1924]. In all experiments the initial concentration of acetoacetate was determined by setting up a control vessel without tissue; this was treated in exactly the same way as the vessels to which tissue slices were added.

The experiments show that added acetoacetic acid disappears anaerobically in all the tissues which have been examined. The rates of disappearance are slightly higher in kidney, liver and pigeon brain than in other tissues, but in testis, rat and guinea-pig brains, spleen, intestine and pigeon pancreas there is a fairly uniform rate, the values of Q_{Acac} being about -1. In rat pancreas, submaxillary gland and diaphragm the rates are distinctly slower. There is acid production in every case.

When substrates are added in addition to acetoacetic acid the following changes occur:

1. In presence of pyruvate there is a marked increase of CO_2 production in all tissues, but acceleration of acetoacetic acid disappearance is constantly observed only in rat liver and in pigeon brain and kidney. In the other tissues examined there is no significant effect on acetoacetic acid reduction.

2. In liver there is a large acid formation in presence of fructose [Oppenheimer, 1912; Dickens & Greville, 1932; Rosenthal, 1930; 1931], and we have found that this is accompanied by a more rapid disappearance of acetoacetic acid. In kidney, where fructolysis is low, the rate of acetoacetic acid dis-

appearance is not altered significantly; likewise there is no effect in testis, brain or other tissues.

3. When *dl*-glyceraldehyde is added to slices of liver, kidney and testis there is acid formation [see Rosenthal, 1930; 1931] and acceleration of acetoacetic acid disappearance equal to that observed with pyruvate. In kidney there is a similar acid formation in presence of methylglyoxal and acetaldehyde, but a smaller effect on disappearance of ketonic acid.

Since glyceraldehyde and methylglyoxal react with acetoacetic acid *in vitro* in absence of tissue, and since the presence of tissue slices causes little or no acceleration of this effect, it is difficult to assess the action of these substances in surviving tissue. We have therefore omitted the $-Q_{\text{Acac}}$ values from Table I.

4. Glucose, galactose, mannose, hexosediphosphate, phosphoglycerate, α-glycerophosphate, glycerol, sorbitol, lactate, acetate, propionate, butyrate, cysteine, succinate and glutathione in the concentrations employed have no significant influence on anaerobic disappearance of acetoacetic acid in the tissues to which they have been added. Alanine, however, was effective in both liver and kidney, whilst α-glycerophosphate increased the rate of disappearance in guinea-pig brain during a short experiment.

Anaerobic disappearance of acetoacetic acid is somewhat slower in the liver of a starved rat but is increased by pyruvate and fructose just as in the well-nourished organ (Exp. 7).

Table II.

(a) *Anaerobic acid formation and acetoacetic acid disappearance in rat liver slices.*

Substrate		$Q_{\text{CO}_2}^{\text{N}_2}$	Q_{Acac}
1. During the first hour:			
Acetoacetic acid, 0·0033 *M*		2·97	-2·16
,,	+ pyruvate, 0·01 *M*	13·5	4·05
,,	+ fructose, 0·01 *M*	11·4	-6·11
2. During the second hour:			
Acetoacetic acid, 0·0033 *M*		2·42	-1·33
,,	+ pyruvate, 0·01 *M*	6·59	-2·65
,,	+ fructose, 0·01 *M*	5·54	-2·91

(b) *Effects of fluoride and iodoacetate on the anaerobic disappearance of acetoacetic acid in liver slices (rat).*

2 hours at 37·5°.

Fluoride. Concentration of NaF = 0·01 *M*. Ca-free medium.			
Acetoacetic acid, 0·003 *M*		1·70	-1·25
,,	+ fluoride	0·98	-1·17
,,	+ pyruvate, 0·01 *M*	6·62	-3·88
,,	+ pyruvate, 0·01 *M* + fluoride	5·70	-3·70
,,	+ fructose, 0·01 *M*	2·56	-2·06
,,	+ fructose, 0·01 *M* + fluoride	1·37	-1·58
Concentration of sodium fluoride = 0·02 *M* Ca-free medium.			
Acetoacetic acid, 0·003 *M*		1·93	-1·18
,,	+ fluoride	1·00	-1·21
,,	+ pyruvate, 0·01	9·82	-3·16
,,	+ pyruvate, 0·01 + fluoride	4·98	-2·55
,,	+ fructose, 0·01	3·95	-2·86
,,	+ fructose, 0·01 + fluoride	1·39	-1·29
Iodoacetate. Concentration of sodium iodoacetate = 0·00067 *M*.			
Acetoacetic acid, 0·003 *M*		2·05	-0·75
,,	+ iodoacetate	1·93	-0·82
,,	+ pyruvate, 0·01 *M*	7·02	-2·36
,,	+ pyruvate, 0·01 *M* + iodoacetate	4·09	-1·57
,,	+ fructose, 0·01 *M*	5·10	-2·27
,,	+ fructose, 0·01 *M* + iodoacetate	2·48	-1·51

The relationship between anaerobic glycolysis and disappearance of acetoacetic acid.

In view of the well-known effects of pyruvate and fructose on anaerobic acid production in liver we performed a number of experiments bearing on the relationship between glycolysis and the reduction of acetoacetic acid (Table II).

Table II (*a*) shows that the rate of acetoacetic acid disappearance diminishes with time in proportion to the fall in fructolysis or in CO_2 production caused by pyruvate. The inhibitors of glycolysis, fluoride and iodoacetate, also inhibit acetoacetic acid disappearance; although fluoride reduces CO_2 formation in presence of pyruvate, it has only a small inhibitory action upon the rate of acetoacetic acid disappearance (Table II (*b*)). Exp. 7 (Table I) illustrates the fact that acetoacetic acid disappearance is slower in the liver of the starved rat than in the well-nourished organ. This experiment also shows that fructolysis and CO_2 formation in presence of pyruvate are less [Rosenthal, 1930; 1931]; in spite of this the substrates still increase acetoacetic acid disappearance. Further, when glucolysis is "activated" by means of 0·001 *M* pyruvate, acetoacetic acid disappearance is not increased in proportion.

These experiments lead to no final conclusions, but they suggest that there is some parallelism between liver glycolysis and acetoacetic acid reduction; the parallelism, however, is not complete in the case of pyruvate.

Aerobic disappearance of acetoacetic acid in animal tissues.

The results of investigation of aerobic disappearance of acetoacetic acid in different tissues and in presence of certain substrates are given in Table III.

Table III. *Aerobic disappearance of acetoacetic acid in presence of tissue slices.*

2–3 ml. phosphate saline, *p*H 7·4. 2 hours at 37·5°. Gas: oxygen.

Animal	Substrate	Q_{O_2}	$Q_{\text{Ac.ac.}}$
	M initial concentration		
	Kidney, cortex.		
Rat	Acetoacetic acid, 0·0033	−29·5	−5·02
	,, +glucose, 0·01	−30·2	−5·55
	,, +pyruvate, 0·01	−33·5	−6·37
Rat	Acetoacetic acid, 0·0033	−27·0	−3·88
	,, +glycerol, 0·01	−27·4	−3·98
Rat	Acetoacetic acid, 0·0033	−22·6	4·33
	,, +α-glycerophosphate, 0·01	−24·6	−4·91
Rat	Acetoacetic acid, 0·0033	−23·6	4·37
	,, +hexosediphosphate, 0·01	−29·9	−5·45
	Testis.		
Rat	Nil	−6·8	+0·51
	Glucose, 0·01	−11·0	+0·56
	Acetoacetic acid, 0·0033	−6·4	−0·28
	,, +glucose, 0·01	−11·3	−0·75
	,, +pyruvate, 0·01	−13·3	−0·34
	,, +fructose, 0·01	10·0	−0·18
	,, +glycerol, 0·01	−6·7	−0·10
Rat	Nil	−5·2	+0·43
	Acetoacetic acid, 0·0033	−6·1	−0·22
	,, +α-glycerophosphate, 0·01	−6·6	−0·17
Rat	Acetoacetic acid, 0·0033	−7·3	−0·18
	,, +hexosediphosphate, 0·01	6·7	0·00
	,, +α-phosphoglycerate, 0·01	−6·5	−0·11
	,, +β-phosphoglycerate, 0·01	−6·1	−0·05
	,, +alanine, 0·01	−5·4	−0·13

Table III (*cont.*).

Animal	Substrate *M* inital concentration	Q_{O_2}	Q_{Acac}
	Brain cortex.		
Guinea-pig	Glucose, 0·01	−11·7	0·00
	Fructose, 0·01	−12·0	0·00
	Pyruvate, 0·01	−14·0	0·00
	Lactate, 0·01	−12·5	0·00
	Acetoacetic acid, 0·0023	−9·1	−1·11
	„ +glucose, 0·01	−12·1	−1·39
	„ +fructose, 0·01	−11·0	−1·04
	„ +pyruvate, 0·01	13·9	−1·22
	„ +lactate, 0·01	−13·0	−1·05
Guinea-pig	Acetoacetic acid, 0·0023	8·6	0·66
	„ +α-glycerophosphate, 0·01	−9·7	−0·99
Pigeon	Nil	−8·9	+0·17
	Pyruvate, 0·01	−18·1	+0·27
	Acetoacetic acid, 0·0033	−16·1	−4·48
	„ +pyruvate, 0·01	−18·6	−3·34
	„ +glucose, 0·01	−14·1	4·72
	Glucose, 0·01	−14·6	+0·20
	Spleen.		
Guinea-pig	Acetoacetic acid, 0·003	−6·8	−1·11
	„ +pyruvate, 0·01	−6·9	−0·90
	Lung.		
Guinea-pig	Nil	−7·4	+0·12
	Acetoacetic acid, 0·003	−8·9	−1·12
	„ +pyruvate, 0·01	−9·0	−0·77
	Pyruvate, 0·01	−8·0	+0·13
	Pancreas.		
Pigeon	Nil	−8·7	+0·18
	Acetoacetic acid, 0·003	−8·5	1·07
	Submaxillary gland.		
Guinea-pig	Nil	−5·0	+0·12
	Acetoacetic acid, 0·003	−7·2	−1·40
	Diaphragm.		
Rat	Acetoacetic acid, 0·0033	−4·8	−1·35
	„ +pyruvate, 0·01	−5·2	1·20
	Skeletal muscle.		
Pigeon	Acetoacetic acid, 0·003	−8·5	−0·60
	„ +pyruvate, 0·01	−11·8	0·91
	„ +fructose, 0·01	13·2	−0·83
	3 ml. bicarbonate-Ringer solution. Gas: 5% CO_2 and 95% O_2.		
	Liver.		
Rat	Nil	—	+0·23
	Pyruvate, 0·01	—	+1·08
	Acetoacetic acid, 0·0023	—	−0·80
	„ +pyruvate, 0·01	—	−0·59
	„ +glucose, 0·01	—	−1·06
	Glucose, 0·01	—	+0·20
	Kidney cortex.		
Rat	Acetoacetic acid, 0·0033	—	−3·57
	„ +pyruvate, 0·01	—	−4·68
	„ +α-phosphoglycerate, 0·01	—	−4·02

The experiments show that the rate of disappearance of added acetoacetic acid is much greater in kidney than in any other tissue except pigeon brain. In testis the disappearance is very small, the figures being within the experimental error, but becomes significant in presence of glucose. In slices of liver and skeletal muscle the rate is slow and unaffected by substrates. In pancreas, diaphragm and submaxillary gland the aerobic disappearance is significantly greater than the anaerobic (see Table I); in spleen it is not. In lung acetoacetic acid disappears aerobically at about the same rate as in spleen.

In all tissues except kidney the addition of substrates produced no acceleration. In kidney pyruvate, α-glycerophosphate, hexosediphosphate, α-phosphoglycerate and glucose caused small but significant increases in rate of acetoacetic acid disappearance.

The oxidation of β-hydroxybutyric acid in various tissues.

Jowett & Quastel [1935] have shown that *dl*-β-hydroxybutyric acid is oxidized to acetoacetic acid by guinea-pig kidney and liver, and by spleen,

Table IV. *Oxidation of β-hydroxybutyric acid in various tissues.*

Phosphate saline, *p*H 7·4. In oxygen at 37·5°.

Animal	Tissue	Substrate (*M*)	Q_{O_2}	Q_{Acac}
Guinea-pig	Brain	Nil	− 6·9	0·49
		dl-β-Hydroxybutyrate, 0·01	− 9·0	1·59
		Nil	− 5·9	0·46
		l-β-Hydroxybutyrate, 0·01	− 6·9	1·92
	Liver	Nil	− 7·8	0·39
		dl-β-Hydroxybutyrate, 0·01	− 8·5	1·28
		Nil	− 8·3	0·91
		l-β-Hydroxybutyrate, 0·01	− 8·8	1·28
	Kidney	Nil	14·0	0·09
		dl-β-Hydroxybutyrate, 0·01	− 20·0	3·25
		Nil	13·2	0·00
		l-β-Hydroxybutyrate, 0·01	− 17·8	2·12
	Spleen	Nil	− 7·9	0·30
		dl-β-Hydroxybutyrate, 0·01	− 9·5	0·84
		Nil	− 8·7	0·17
		l-β-Hydroxybutyrate, 0·01	− 9·8	0·73
	Pancreas	Nil	− 2·90	0·00
		dl-β-Hydroxybutyrate, 0·01	− 2·94	0·28
	Submaxillary gland	Nil	− 5·0	0·12
		dl-β-Hydroxybutyrate, 0·01	− 8·5	0·65
	Skeletal muscle	Nil	− 2·12	0·19
		dl-β-Hydroxybutyrate, 0·01	− 2·36	0·18
	Blood 2·0 ml. (citrated)	Nil	—	0·00
		dl-β-Hydroxybutyrate, 0·01	—	0·00
Rat	Kidney	Nil	− 17·7	0·10
		l-β-Hydroxybutyrate, 0·005	− 22·3	1·43
	Lung	Nil	− 7·9	0·28
		dl-β-Hydroxybutyrate, 0·01	− 8·3	0·34
	Intestine	Nil	− 7·1	0·20
		l-β-Hydroxybutyrate, 0·01	− 6·3	0·53
	Pancreas	Nil	− 3·7	0·02
		dl-β-Hydroxybutyrate, 0·01	− 4·1	0·11
	Diaphragm	Nil	− 5·5	0·16
		dl-β-Hydroxybutyrate, 0·01	− 5·3	0·36
		Nil	− 5·3	0·25
		l-β-Hydroxybutyrate, 0·01	− 4·7	0·66
Pigeon	Pancreas	Nil	− 8·7	0·18
		dl-β-Hydroxybutyrate, 0·01	− 9·0	0·53
	Skeletal muscle	Nil	8·5	0·10
		dl-β-Hydroxybutyrate, 0·01	− 11·7	0·15

NOTE. *l*-β-Hydroxybutyrate was prepared from the Ca Zn salt by decomposition with sodium carbonate.

testis and brain cortex of the rat. We have confirmed these observations and found that the oxidation takes place at approximately the same rate in presence of the same concentration of *l*-β-hydroxybutyric acid (Table IV).

The oxidation also occurs in other tissues: in pancreas and submaxillary gland of the guinea-pig, in intestine and diaphragm of the rat and in pigeon pancreas. Though the values of Q_{Acac} are small, the differences are significant. Guinea-pig blood and rat lung slices do not appear to oxidize β-hydroxybutyric acid.

As a result of the experiments of this and preceding sections it becomes clear that the reaction, acetoacetic acid $\rightleftharpoons$ β-hydroxybutyric acid, is reversible and can occur in tissues other than liver and kidney, although the respective rates of oxidation and reduction vary widely in different tissues.

The aerobic destruction of β-hydroxybutyric acid.

By determining acetoacetic and β-hydroxybutyric acids simultaneously we have measured the real destruction of β-hydroxybutyric acid as distinct from the conversion into ketonic acid. This has been done chiefly in kidney in presence and in absence of added substrates, but a few experiments were made with other tissues (Table V).

Table V. *Aerobic breakdown of β-hydroxybutyric acid.*

Phosphate saline and oxygen. 37·5°.

	Substrate Initial conc. 0·005 *M*, β-hydroxybutyrate		Q_{O_2}	Q_{Acac}	$Q_{\beta\text{-Hydroxy}}$
Rat kidney	Nil		−17·8	0·0	0 0
	dl-β-Hydroxybutyric acid		−23·9	+2·80	−5·90
	dl-β-Hydroxybutyric acid		31·6	+1·32	5·34
	,,	+succinate, 0·01 *M*	−45·7	+1·27	5·73
	,,	+glucose, 0·01 *M*	−33·6	+1·30	−5·30
	dl-β-Hydroxybutyric acid		−23·0	+0·45	−5·17
	,,	+fructose, 0·01 *M*	−32·0	+0·20	−5·20
	dl-β-Hydroxybutyric acid	+pyruvate, 0·01 *M*	−32·02	—	5·22
	dl-β-Hydroxybutyric acid		−28·3	+0·90	−4·44
	,,	+glycerol, 0·01 *M*	−32·6	+0·71	4·93
	,,	+lactate, 0·01 *M*	−29·0	+0·76	3·64
	dl-β-Hydroxybutyric acid		−25·7	+0·95	−5·51
	,,	+hexosediphosphate 0·01 *M*	−29·0	+0·90	−5·04
	10 ml. phosphate saline and O_2. Large shaking vessels.				
Rat testis	*dl*-β-Hydroxybutyric acid		—	+0·55	−1·06
	,,		—	+0·45	−1·08
	dl-β-Hydroxybutyric acid	+succinate, 0·01 *M*	—	+0·67	−1·95
	,,	+glucose, 0·01 *M*	—	+0·31	−1·68
Rat lung	*dl*-β-Hydroxybutyric acid		—	+0·05	−1·10
	,,		—	+0·05	−1·15

These figures confirm the findings of Quastel & Wheatley [1935], who observed that only about one-quarter of the β-hydroxybutyric acid disappearing in kidney is converted into acetoacetic acid. They show further that addition of substrates such as glucose, succinate and pyruvate does not increase the rate of breakdown. In absence of other added substrates β-hydroxybutyric acid is broken down at an appreciable rate in rat testis and lung; in the former tissue glucose does not accelerate the process.

The influence of inhibitors on ketone body disappearance.

Quastel & Wheatley [1935] have shown that sodium malonate greatly inhibits the disappearance of acetoacetic acid in kidney slices, an effect which occurs only aerobically. This we have confirmed and also the action of fumarate and lactate in preventing inhibition. The ketogenic effect of malonate has been referred to in Part III of this series (see also Jowett & Quastel [1935]), where it was shown that other dicarboxylic acids—hydroxymalonic, mesoxalic, tartaric

Table VI. *Influence of anticatalysts on the disappearance of ketone bodies in rat kidney and liver.*

2 hours at 37·5°.

Substrate	Q_{O_2}	$Q_{\text{Ac.ac.}}$
Kidney.		
Aerobic. Bicarbonate-Ringer solution: 5% CO_2 and 95% O_2.		
Acetoacetic acid, 0·003 *M*	—	- 3·28
„ + malonate, 0·02 *M*	—	- 0·27
„ + hydroxymalonate 0·02 *M*	—	- 2·10
„ + NH_4Cl, 0·02 *M*	—	3·12
„ + pyruvate, 0·01 *M*	—	- 3·70
„ + pyruvate, 0·01 *M* + malonate, 0·02 *M*	—	- 1·13
Acetoacetic acid, 0·003 *M*	—	3·50
„ + oxalate, 0·003 *M*	—	- 3·23
Aerobic. Phosphate saline and oxygen.		
Acetoacetic acid, 0·003	30·6	3·88
„ + NH_4Cl, 0·01 *M*	29·8	- 3·80
Nil	- 22·2	+ 0·49
Malonate, 0·01 *M*	14·7	+ 1·09
Hydroxymalonate, 0·01 *M*	- 20·6	+ 0·47
Butyrate, 0·01 *M*	29·8	+ 0·79
„ + malonate, 0·01 *M*	- 19·4	+ 1·24
„ + hydroxymalonate, 0·01 *M*	23·3	+ 0·52
Nil	- 20·1	- 0·17
Oxalate, 0·003 *M*	- 18·3	+ 0·06
Butyrate, 0·01 *M* + oxalate, 0·003 *M*	29·8	- 0·33
Nil	22·2	+ 0·32
Acetoacetic acid, 0·0023 *M*	- 25·8	3·65
NH_4Cl, 0·01 *M*	20·3	0·00
NH_4Cl, 0·02 *M*	- 16·1	0·00
Acetoacetic acid, 0·0023 *M* + NH_4Cl, 0·01 *M*	27·4	- 3·53
„ + NH_4Cl, 0·02 *M*	- 23·5	- 3·26
Malonate, 0·02 *M*	- 9·0	+ 0·57
Hydroxymalonate, 0·02 *M*	- 19·4	0·00
Acetoacetic acid, 0·0023 *M* + malonate, 0·02 *M*	- 8·6	0·31
„ + hydroxymalonate, 0·02 *M*	21·9	- 2·88
Nil	- 20·4	+ 0·25
d-Tartrate, 0·01 *M*	- 19·6	+ 0·10
Acetoacetic acid, 0·0023 *M*	25·3	- 3·33
„ + *d*-tartrate, 0·01 *M*	- 24·4	- 3·30
„ + *d*-tartrate, 0·02 *M*	- 23·0	- 3·02
Nil	22·6	+ 0·23
Oxalate, 0·005 *M*	- 19·8	0·00
Acetoacetic acid, 0·0023 *M*	- 30·6	4·41
„ + oxalate, 0·005 *M*	- 29·1	- 4·03
„ + oxalate, 0·01 *M*	29·2	3·90
Nil	22·2	+ 0·31
Mesoxalate, 0·01 *M*	- 22·6	+ 0·11
Mesoxalate, 0·02 *M*	19·2	+ 0·09
Acetoacetic acid 0·0023 *M*	- 24·3	- 3·02
„ + mesoxalate, 0·01 *M*	- 22·8	- 3·15
„ + mesoxalate, 0·02 *M*	18·3	- 2·31

Table VI (*cont.*).

2 hours at 37·5°.

Substrate	Q_{CO_2}	Q_{Acac}
Liver.		
Anaerobic. Bicarbonate-Ringer solution; 5 % CO_2 and 95 % N_2.		
Acetoacetic acid, 0·0023 *M*	2·23	-1·36
„ + malonate, 0·01 *M*	3·18	1·41
„ + hydroxymalonate, 0·01 *M*	3·47	-1·67
„ + NH_4Cl, 0·02 *M*	1·91	1·27
Acetoacetic acid, 0·0023 *M*	1·26	-0·95
„ + oxalate, 0·003 *M*	0·99	-1·05
Nil	2·74	+0·03
NH_4Cl, 0·02	2·94	+0·02
Acetoacetic acid, 0·0023 *M*	2·04	-1·11
„ + NH_4Cl, 0·02 *M*	1·87	1·64
„ + pyruvate, 0·01 *M*	5·56	-2·63
„ + pyruvate, 0·01 *M* + NH_4Cl, 0·02 *M*	5·79	2·32

NOTE. Experiments with oxalate were performed in Ca-free media.

and oxalic—are ketogenic in liver. Since these substances take no direct part in fatty acid metabolism, and since they appear to inhibit some dehydrogenase systems more or less specifically, they may be regarded as anticatalysts. Ammonium chloride possibly belongs to the same class. We have examined the influence of these substances on ketone body disappearance in kidney, the results being given in Table VI.

It will be seen that malonate is a powerful and relatively specific inhibitor of respiration and of aerobic disappearance of acetoacetic acid in kidney. Hydroxymalonate, mesoxalate, tartrate, oxalate and ammonia cause relatively little depression of respiration and only a small inhibition of acetoacetic acid disappearance when they are added in appropriate concentrations. Pyruvate prevents the malonate action to some degree.

In liver under anaerobic conditions these substances do not significantly affect the rate of acetoacetic acid disappearance.

The influence of malonate and hydroxymalonate on the oxidation of *β*-hydroxbutyric acid was investigated (Table VII).

Table VII. *Influence of malonate and hydroxymalonate on oxidation of β-hydroxybutyric acid.*

Rat kidney cortex. Phosphate saline and oxygen.

Substrate	Q_{O_2}	Q_{Acac}	$Q_{\beta\ Hydroxy}$
dl-*β*-Hydroxybutyric acid, 0·005 *M*	-25·0	+0·95	-6·80
„ + malonate, 0·01 *M*	-18·1	+0·67	-4·50
dl-*β*-Hydroxybutyric acid, 0·0075 *M*	-41·7	+3·17	—
„ + malonate, 0·01 *M*	-14·2	+3·16	—
„ + malonate, 0·02 *M*	-11·0	+3·00	—
dl-*β*-Hydroxybutyric acid, 0·005 *M*	-34·3	+2·21	—
„ + hydroxymalonate, 0·02 *M*	-27·8	+2·05	—

These figures show that malonate and hydroxymalonate do not inhibit the oxidation of *β*-hydroxybutyric acid to acetoacetic acid; and that malonate prevents the aerobic breakdown of *β*-hydroxybutyric acid.

DISCUSSION.

A considerable portion of the acetoacetic acid or β-hydroxybutyric acid added to kidney slices is broken down to products no longer recognizable as ketone bodies. At least part of this disappearance is due to complete combustion, since Elliott *et al.* [1935] have found that bicarbonate is formed during oxidation of β-hydroxybutyric acid, but there may be intermediate stages.

The apparently specific action of malonate in preventing aerobic breakdown of ketone bodies acquires further significance from the fact that malonate is known to be a specific inhibitor of succinic dehydrogenase. As a tentative working hypothesis we suggest that the aerobic metabolism of ketone bodies may depend upon the oxidation of succinic acid.

Krebs [1936] has described the reduction of acetoacetic acid as a linked reaction which involves the anaerobic oxidation of carbohydrate derivatives. This provides one point of contact between fat and carbohydrate metabolism.

Another is possible. If acetic acid were formed by cleavage of ketone bodies, it might react with pyruvic acid according to Krebs's scheme:

$$\begin{array}{c}\text{Acetic acid}\\ +\\ \text{pyruvic acid}\end{array} \rightarrow \alpha\text{-Ketoglutaric acid} \rightarrow \text{Succinic acid, etc.}$$

The continuation of ketone body breakdown would then depend upon the oxidation of succinic acid and the consequent supply of pyruvic acid.

The action of malonate could be explained as an interruption of the chain of reactions leading to pyruvic acid.

$$\text{Succinic acid} \xrightarrow{*} \text{Fumaric acid} \rightarrow \text{Malic acid} \rightarrow \text{Oxaloacetic acid} \rightarrow \text{Pyruvic acid.}$$

* Malonate inhibition.

The partial neutralization of the malonate effect by fumarate, lactate, alanine [Quastel & Wheatley, 1935] and pyruvate agrees with this hypothesis.

Preliminary measurements of acid-base changes have shown that, within the limits of error, (i) the rate of bicarbonate formation in kidney in presence of ketone bodies is equal to or greater than the rate of ketone body destruction, (ii) acetate is burnt approximately twice as rapidly as the ketone bodies. These observations are consistent with the view that ketone bodies are split into acetic acid before combustion, but they afford no proof.

SUMMARY.

1. The metabolism of ketone bodies, both aerobic and anaerobic, has been investigated in a number of tissues by means of the slice technique. The rate of metabolism varies in different tissues.

2. Pyruvate and fructose accelerate the anaerobic disappearance of acetoacetic acid in liver but have no marked influence in other tissues except pigeon's kidney.

3. Since β-hydroxybutyric acid is oxidized to acetoacetic acid by most tissues, the reaction, acetoacetic acid $\rightleftharpoons$ β-hydroxybutyric acid, appears to be of general importance.

4. The effects of malonate, hydroxymalonate, mesoxalate, tartrate and oxalate on ketone body oxidation have been studied.

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